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PRINCIPAL INVESTIGATOR: David Crowe, Ph.D.

CONTRACTING ORGANIZATION: University of Illinois
Chicago, IL 60612

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David Crowe, Ph.D.

E-Mail: dlcrowe@uic.edu

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14. ABSTRACT
We proposed that alterations in histone methylation regulate MSC fate commitment and predispose these progeny to malignant transformation. Transformed ER+ epithelial cells deregulate proliferation of MSC and luminal progenitors contributing to transformation of ER- luminal and basal cells and development of treatment resistant breast cancer. We previously reported that transformed MSC with reduced DNA repair contribute to more aggressive mammary tumors. Transformed luminal progenitor cells with altered histone methylation produced aggressive mammary tumors after long latency. The fractions of progenitor and differentiated cells in these tumors also were altered. ER+ tumor cells promoted proliferation and metastasis of tumor derived MSC. H3K27me3 levels were increased in JMJD3 depleted ER+ tumor cells. Tumors derived from MMTV-Wnt1 MSC co-transplanted with JMJD3 shRNA transduced ER+ cells demonstrated increased latency with decreased tumor number, volume, and metastasis. Tumors derived from MMTV-Wnt1 MSC co-transplanted with JMJD3 shRNA transduced ER+ cells were classified as poorly differentiated adenocarcinoma by histopathologic analysis. The MSC fraction was reduced in tumors containing JMJD3 shRNA transduced ER+ cells compared to control transduced tumors. The implications of the results presented in this report suggest that while tumor suppressor pathways can inhibit mammary tumorigenesis when DNA damage repair is inhibited, more aggressive clones may eventually evolve via genomic instability with the ability to proliferate and metastasize. Decreased DNA damage repair or altered epigenetic marks can dramatically affect the cellular composition of these tumors, thereby regulating phenotype and outcomes. Paracrine factors secreted by ER+ tumor cells can induce proliferation of tumorigenic MSC, leading to increased genomic instability and metastasis. Epigenetic marks in specific tumor cell populations (MSC vs. ER+) can dramatically alter tumor phenotype and outcomes.

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INTRODUCTION

Breast cancer and its treatment impose significant physical, psychological, and economic burdens on patients, their families, and society. African-American women under age 35 have the highest age adjusted mortality rates from breast cancer. The incidence of breast cancer in this group also is higher than for Caucasian women. In several studies these disparities persisted after other variables such as socioeconomic status and access to care were controlled. These studies point to biological differences in breast cancer among these populations such as increased prevalence of the high grade, estrogen receptor negative, basal subtype of the disease in African-American women. Targeting estrogen receptor α (ER) action is a classic example of molecular therapy for breast cancer (for review see Russo et al., 2000; Crowe, 2002). ER- mammary stem cells (MSC) are believed to be the progenitor population for all breast epithelia including acinar cells and the largely ER- basal and luminal cells of the ducts from which most breast cancer arises (for review see Crowe et al., 2004; Visvader and Lindeman, 2006; Sleeman et al., 2007). The luminal epithelial population also contains a subpopulation of ER- progenitors (Asselin-Labat et al., 2007). ER is expressed by a fraction of luminal epithelial cells in normal mammary glands, but the numbers of these cells often are greatly increased in human breast cancer. MSC can reconstitute mammary glands and have been isolated from both humans and mice using cell surface markers (Gudjonsson et al., 2002; Dontu et al., 2003; Stingl et al., 2006; Shackleton et al., 2006). Some of these MSC populations are expanded in mouse mammary cancer models (Li et al., 2003; Liu et al., 2004), and tumorigenic progenitor populations have been isolated from human breast cancers (Al-Hajj et al., 2003; Ponti et al., 2005). These studies have demonstrated an important role for MSC in mammary gland development and tumorigenesis. However these models have not determined how MSC or luminal progenitors give rise to ER+ epithelial cells during mammary gland development, what predisposes ER+ cells to malignant transformation, and how these cells regulate breast tumorigenesis in conjunction with transformed ER- MSC and luminal progenitors. In clinical studies, EZH2 histone methyltransferase (HMT) expression has been associated with poorly differentiated and aggressive breast cancer in humans (Kleer et al., 2003; Raaphorst et al., 2003; Bachmann et al., 2006; Collett et al., 2006; Ding et al., 2006). The histone demethylase JMJD3 reverses the EZH2 mediated histone H3 lysine-27 methyl mark. We proposed a new mechanism of breast tumorigenesis that, during normal mammary gland development, decreased expression of DNA repair genes during HMT mediated differentiation of MSC to ER+ luminal cells makes the latter population more susceptible to transformation and expansion. These transformed cells may induce aberrant proliferation of the ER- MSC or luminal progenitors resulting in genomic instability, production of additional transformed ER+ or ER- luminal cells, or transformation of MSC giving rise to the aggressive basal subtype of mammary cancer. We hypothesized that alterations in histone methylation regulate MSC fate commitment and predispose these progeny to malignant transformation. Transformed ER+ epithelial cells deregulate proliferation of MSC and luminal progenitors contributing to transformation of ER- luminal and basal cells and development of treatment resistant breast cancer. **Specific Aim #1** will test the hypothesis that EZH2 regulates both ER and DNA repair gene expression in MSC resulting in the differentiation of ER+ but transformation sensitive mammary epithelial cells. Specific Aim #1 will test this hypothesis by comparing transformation of these cell populations following altered histone methylation. **Specific Aim #2** will test the hypothesis that transformed ER+ cells regulate proliferation and tumorigenicity of the MSC and luminal progenitor populations by determining the molecular and cellular effects of co-transplantation of these populations. Understanding the relationships between normal and transformed mammary epithelial cells has important ramifications for preventing ER- and basal subtype breast cancer in African-American women. The biologically aggressive basal subtype of breast cancer may result from transformation of the less differentiated MSC population. Targeting interactions between ER+ cells and MSC to prevent progression to these treatment resistant tumors will improve survival and quality of life.

BODY

The following summary of results was detailed in previous progress reports:

We have characterized tumors derived from transformed mammary stem cells (MSC) with reduced Rad50 and NBS1 expression and CD61+ luminal progenitors with reduced EZH2 expression. These transplanted tumors were characterized as poorly differentiated adenocarcinoma by histopathology. Tumors with reduced Rad50/NBS1 or EZH2 expression demonstrated markedly increased latency (mean 72 and 78 weeks respectively; $P<0.01$). However these tumors were highly metastatic compared to those derived from control clones (4 fold increase in lung tumors). These tumors were analyzed for cellular composition by flow cytometry. Compared to tumors derived from control clones, those with decreased Rad50/NBS1 expression exhibited a 2 fold increase in the percentage of MSCs ($P<0.05$). The number of estrogen receptor (ER) positive cells in these tumors was reduced by 9 fold ($P<0.03$). These results indicate that mammary tumors derived from transformed MSC with reduced DNA repair have increased latency and metastasis and reduced ER+ differentiation.

We characterized mammary tumors derived from MMTV-Wnt1 MSC transplanted with ER+ or ER- tumor cells to cleared mammary fat pads of nude mice. These transplanted tumors were characterized as poorly differentiated adenocarcinoma by histopathology. Transplanted tumors derived from MMTV-Wnt1 MSC and ER+ tumor cells exhibited decreased latency compared to those transplanted with MSC and ER- tumor cells (4 vs. 12 weeks respectively; $P<0.03$). MSC/ER+ transplants developed a significantly higher number of tumors than MSC/ER- transplants (19 versus 11 tumors; $P<0.05$). MSC/ER+ tumors exhibited a higher percentage of proliferating cells (52% versus 21%; $P<0.01$). MSC/ER+ tumors were highly metastatic compared to MSC/ER- tumors (35% versus 12% metastatic tumors; $P<0.004$). MSC/ER+ tumors exhibited a higher percentage of MSC (40% versus 21%; $P<0.04$). These results indicate that tumor derived ER+ cells enhance proliferation and tumorigenicity of transplanted tumorigenic MSC.

The following results were obtained since the previous progress report:

We inhibited JMJD3 expression in ER+ tumor cells by lentiviral transduction followed by puromycin selection. Reduced JMJD3 expression compared to control transduced cells is shown by qRT-PCR in Fig. 1. H3K27me3 levels were correspondingly increased in JMJD3 depleted cells as shown by western blot (Fig. 2). JMJD3 shRNA transduced cells were co-transplanted with tumorigenic MSC to cleared mammary fat pads. Tumors demonstrated increased latency (8 vs. 3 weeks; $P<0.0001$) with decreased tumor number (9 vs. 20; $P<0.00001$), volume (46 vs. 125 mm^3; $P<0.002$), and metastasis (10% vs. 50%; $P<0.005$; Fig. 3A-D). Tumors were classified as poorly differentiated adenocarcinoma by histopathologic analysis (Fig. 4A, B). We demonstrated MSC in tissue sections using CD24/CD49f immunofluorescence (Fig. 4C). The MSC fraction was reduced in tumors containing JMJD3 shRNA transduced ER+ cells compared to control transduced tumors (16% vs. 39%; $P<0.0002$; Fig. 5). These results indicate that altering epigenetic marks in specific tumor cell populations may have different effects on tumor biology and outcomes.

KEY RESEARCH ACCOMPLISHMENTS

The following research accomplishments were detailed in previous progress reports:

- Tumors derived from MSC with reduced Rad50 and NBS1 expression and CD61+ luminal progenitors with reduced EZH2 expression were characterized as poorly differentiated adenocarcinoma by histopathology.
- Tumors with reduced Rad50/NBS1 or EZH2 expression demonstrated markedly increased latency.
- Tumors with reduced Rad50/NBS1 were highly metastatic compared to those derived from control clones.
- Tumors with decreased Rad50/NBS1 expression exhibited a 2 fold increase in the percentage of MSCs. The number of estrogen receptor (ER) positive cells in these tumors was reduced by 9 fold.
- Transplanted tumors derived from MMTV-Wnt1 MSC and ER+ or ER- tumor cells were characterized as poorly differentiated adenocarcinoma by histopathology.
- Transplanted tumors derived from MMTV-Wnt1 MSC and ER+ tumor cells exhibited decreased latency compared to those transplanted with MSC and ER- tumor cells.
- MSC/ER+ transplants developed a significantly higher number of tumors than MSC/ER- transplants.
- MSC/ER+ tumors exhibited a higher percentage of proliferating cells.
- MSC/ER+ tumors were highly metastatic compared to MSC/ER- tumors.
- MSC/ER+ tumors exhibited a higher percentage of MSC.

The following research accomplishments were obtained since the previous progress report:

- H3K27me3 levels were increased in JMJD3 depleted ER+ tumor cells.
- Tumors derived from MMTV-Wnt1 MSC co-transplanted with JMJD3 shRNA transduced ER+ cells demonstrated increased latency with decreased tumor number, volume, and metastasis.
- Tumors derived from MMTV-Wnt1 MSC co-transplanted with JMJD3 shRNA transduced ER+ cells were classified as poorly differentiated adenocarcinoma by histopathologic analysis.
- The MSC fraction was reduced in tumors containing JMJD3 shRNA transduced ER+ cells compared to control transduced tumors.

REPORTABLE OUTCOMES

An abstract was presented at the 2011 Era of Hope breast cancer research teeting in Orlando, FL. Two manuscripts were published which are referenced in this section. The abstract and publications are included in the appendix.

Abstract:

Crowe DL, Wan R, Baloue K. 2011. Coordinate activation of the NBS1 gene regulates estradiol mediated protection from double strand DNA breaks. Era of Hope Breast Cancer Research Meeting. Orlando, FL

Published manuscripts:

Wan R, Crowe DL. 2012. Haploinsufficiency of the Nijmegen breakage syndrome 1 gene increases mammary tumor latency and metastasis. Int J Oncol 41:345-352

Wan R, Wu J, Baloue KK, Crowe DL. 2013. Regulation of the Nijmegen breakage syndrome 1 gene NBS1 by c-my, p53, and coactivators mediates estrogen protection from DNA damage in breast cancer cells. Int J Oncol 42:712-720

The principal investigator Dr. David L. Crowe and co-investigator Dr. Jianchun Wu received salary support from the current award.

CONCLUSIONS

The implications of the results presented in this progress report suggest that while tumor suppressor pathways can inhibit mammary tumorigenesis when DNA damage repair is inhibited, more aggressive clones may eventually evolve via genomic instability with the ability to proliferate and metastasize. Decreased DNA damage repair or altered epigenetic marks can dramatically affect the cellular composition of these tumors, thereby regulating phenotype. Paracrine factors secreted by ER+ tumor cells can induce proliferation of tumorigenic MSC, leading to increased genomic instability and metastasis. These results also indicate that epigenetic marks in specific tumor cell populations (MSC vs. ER+) can dramatically alter tumor phenotype and outcomes.

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APPENDIX

Abstract presented at 2011 Era of Hope Meeting:

COORDINATE ACTIVATION OF THE NBS1 GENE REGULATES ESTRADIOL-MEDIATED PROTECTION FROM DOUBLE-STRAND DNA BREAKS

David Crowe, Rowena Wan, and Kaitrin Baloue

University of Illinois, Chicago, IL

Introduction: Double-strand break repair is mediated by two major repair pathways, homologous recombination (HR) or nonhomologous end joining (NHEJ). In mammalian cells more than 90% of double-strand breaks are repaired by NHEJ. Impairment of these pathways is associated with cell cycle arrest, cell death, genomic instability, and cancer. Human diseases such as Nijmegen breakage syndrome, due to mutations in the NBS1 gene, produce defects in resection of double-strand breaks. NBS1 polymorphisms have been associated with increased risk of breast cancer. We previously demonstrated that estradiol protected estrogen receptor (ER)-positive breast cancer cell lines against double-strand breaks and cell death.

Objective: To determine the mechanism by which ER-positive breast cancer cells are protected from double-strand breaks and cell death.

Methods: Human mammary epithelial and breast cancer cell lines were cultured in DMEM and 10% fetal bovine serum. Primary cultures of NBS1+/-;neu and NBS1+/+;neu mouse mammary tumor cells were established by trypsinization of minced tumor tissue and cultured in this medium. Human breast cancer cell lines were treated with estradiol (E2), ionizing radiation (IR), combined E2 and radiation, or vehicle. Cells were transfected with c-myc, CBP, SRC1, or neomycin resistance plasmids. For gene knockdown experiments, cells were transfected with shRNA. DNA damage was quantitated by single cell gel electrophoresis. Apoptotic cells were determined by TUNEL assay. Gene expression was determined by quantitative reverse transcriptase-polymerase chain reaction and western blot. Occupancy of the NBS1 intron 1 by p53, c-myc, coactivators, or histones was determined by chromatin immunoprecipitation and nucleosomal mapping. Intron activity and double-strand break repair was determined by reporter gene analysis. Tumor development in NBS1+/-;neu mice was compared to that in NBS1+/+;neu control mice. Mammary tumor tissue was processed for histopathologic and molecular analysis.

Results: The combination of E2 and IR was required to induce NBS1 expression in ER-positive breast cancer cell lines. The protective effect of E2 against double-strand break damage was dependent on ER expression. NBS1 mediated the E2 protective effects against ionizing radiation-induced double-strand break damage and apoptosis. E2 and IR were required to activate the NBS1 intron 1 via cooperative c-myc and p53 binding to their cognate binding sites. E2 and IR recruited coactivators SRC1 and CBP to the myc and p53 binding sites of the NBS1 intron 1. Coactivator induction of NBS1 gene expression was dependent on these sites, and c-myc functionally substituted for E2 treatment in ER-positive cells. CBP and SRC1 functionally substituted for both E2 and IR induction of NBS1 gene expression. ER-positive cells in oncogene driven mammary tumors exhibited fewer double-strand breaks than ER-negative cells. NBS1 haploinsufficiency produced increased double-strand breaks and inhibited oncogene driven mammary tumorigenesis via induction of apoptotic cell

death. However, tumors arising in this model were highly metastatic as the result of increased genetic alterations in transformed mammary epithelial cells.

Published manuscripts:

INTERNATIONAL JOURNAL OF ONCOLOGY 41: 345-352, 2012

Haploinsufficiency of the Nijmegen breakage syndrome 1 gene increases mammary tumor latency and metastasis

ROWENA WAN and DAVID L. CROWE

University of Illinois Cancer Center, Chicago, IL 60612, USA

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Abstract. Human diseases such as Nijmegen breakage syndrome due to mutations in the NBS1 gene result in defects in resection of double strand breaks. NBS1 functions as part of the MRN complex which functions in homologous recombination and non-homologous end joining. NBS is a rare human autosomal recessive disorder caused by hypomorphic mutations. At the cellular level, NBS is characterized by radiosensitivity, chromosomal breakage and defective cell cycle checkpoints. NBS1 null mutations result in early embryonic lethality in mice, but NBS1 hypomorphic mutants are viable. Cells from these mice are defective in S phase and G2/M checkpoints. In humans, NBS1 polymorphisms have been associated with increased risk of breast cancer. MRN expression was reduced in the majority of breast tumors, and low expression of MRN correlated with increased histologic grade and estrogen receptor negativity. While these studies have shown NBS1 to be important in clinical outcomes of patients with breast cancer, mammary tumors are rare in the NBS1 haploinsufficient mouse. To better understand the role of NBS1 in mammary tumorigenesis, we examined the NBS1+/-;MMTV-neu mouse model. Mammary tumor latency was markedly increased in NBS1+/-;neu mice compared to NBS1+/+;neu control animals. This effect was due to increased apoptosis in early NBS1+/-;neu mammary tumors. However, NBS1+/-;neu mammary tumors were highly metastatic and demonstrated clear differences in gene expression profiles compared to control tumors. We concluded that NBS1 haploinsufficiency results in increased mammary tumor latency and metastasis.

Introduction

Double strand break repair is mediated by two major repair pathways, homologous recombination (HR) or non-homologous end joining (NHEJ) (1). In mammalian cells more than 90% of double strand breaks are repaired by NHEJ. Both pathways are defined and their impairment is associated with cell cycle arrest, cell death, genomic instability, and cancer (2). Human diseases such as Nijmegen breakage syndrome due to mutations in the NBS1 gene result in defects in resection of double strand breaks (3). NBS1 functions as part of the MRN complex whose functions are not restricted to HR but are also involved NHEJ (4).

NBS is a rare human autosomal recessive disorder caused by hypomorphic mutations. This disorder is characterized by growth retardation, immunodeficiency, microcephaly, and cancer predisposition. At the cellular level, NBS is characterized by radiosensitivity, chromosomal breakage, and defective cell cycle checkpoints. NBS1 null mutations in mice result in early embryonic lethality (5), but NBS1 hypomorphic mutants are viable (6). Cells from these mice are defective in S phase and G2/M checkpoints. Heterozygous mice with an NBS1 null mutation and homozygous animals with hypomorphic mutations are predisposed to different types of cancer. Conditional NBS1 mutant mice also have been characterized (7). For example, neuronal inactivation of NBS1 results in chromosomal breaks, microcephaly, growth retardation, cerebellar defects, and ataxia.

NBS1 polymorphisms have been associated with increased risk of breast cancer (8-10). MRN expression was reduced in the majority of breast tumors (11), and low expression of MRN correlated with increased histologic grade and estrogen receptor negativity. Response to radiotherapy correlated with high expression of the MRN complex. Patients with high numbers of ionizing radiation induced NBS1 foci had aggressive breast cancer phenotypes (12,13). While these studies have shown NBS1 to be important in clinical outcomes of patients with breast cancer, mammary tumors are rare in the NBS1 haploinsufficient mouse. To better understand the role of NBS1 in mammary tumorigenesis, we characterized these cancers using the NBS1+/-;MMTV-neu mouse.

Materials and methods

Transgenic mouse procedures. Animal procedures were approved by the Institutional Animal Care Committee NBS1+/- mice (6) were crossed with the mammary tumor prone MMTV-neu transgenic strain in the FVB background (The Jackson Laboratory, Bar Harbor, ME). Tumor development in 30 NBS1+/-;neu mice was compared to that in 30 NBS1+/+;neu

Correspondence to: Dr David L. Crowe, University of Illinois Cancer Center, 801 S. Paulina St., Room 530C, Chicago, IL 60612, USA
E-mail: dlcrowe@uic.edu

Key words: Rad50, Mre11, Nijmegen breakage syndrome 1 gene, MMTV-neu, DNA damage

control mice. Mice were genotyped by PCR analysis of tail DNA samples. The mammary gland chains of female mice were examined visually and by palpation twice weekly. Tumors were measured twice weekly using calipers. Mice were euthanized 4 or 8 weeks after tumor formation followed by complete necropsy. Tumor tissue was processed for histopathologic and gene expression analyses. Statistical analysis was determined by ANOVA.

Mammary gland and tumor histopathology. For whole mount analysis, mammary glands were fixed in 3:1 ethanol:acetic acid, mounted on microscope slides, and stained with carmine-alum followed by clearing in 2:1 benzyl benzoate:benzyl alcohol. Tumor tissue was fixed in formalin for 16 h at room temperature. Tissue was dehydrated in an ethanol series followed by clearing in xylene and embedding in paraffin. Five micrometer sections were cut from the blocks and placed on poly-L-lysine coated slides. Sections were deparaffinized in xylene and stained with hematoxylin and eosin for histopathologic interpretation.

Cellular proliferation and programmed cell death analysis. Mouse mammary tumor tissue was dissociated to single cells by trypsinization, fixed with 2% formaldehyde for 20 min at 4°C followed by permeabilization with 70% ethanol, then washed with PBS. For proliferation analysis, cells were incubated with anti-PCNA antibody followed by anti-rabbit IgG secondary antibody conjugated to fluorescein. Cells were washed extensively with PBS. For programmed cell death analysis, cells were incubated with terminal deoxynucleotidyl transferase and fluorescein conjugated dUTP at 37°C for 30 min followed by washing in PBS. The percentage of fluorescein positive cells in each group was determined by flow cytometry.

Reverse transcription-polymerase chain reaction. RNA was extracted from mouse mammary tumors using a commercially available kit (Qiagen, Valencia, CA) and reverse transcribed using SuperScript II reverse transcriptase according to manufacturer's instructions (Invitrogen, Carlsbad, CA). cDNA was amplified using specific primers (5'-3'): Nbs1, TTCCCCCAT ACAAGTATCCAG and AACTTAAGGAGCATCTATGCAG; Malat1, CATCCCGTTCCTTGTTACTC and AGACTACAA ACATTGTGTCGTG; Cdkn2b, GCTAAATGGGAAACC TGGAGAG and ACGTTGAGTCTGTCAGAATCC; Hif1a, CAGCTCCCTTTCTGATAAGC and TCTTCAGTTTCTGT GTCATCG; Egf, TCTGTCAACCCCTGAATAAATG and TTTCACTGGGAAAGACTTCAAG; Lalba, TACCCTGTA GTGACACCACC and TAAAACCCCCATCGAGACC; Jag1, AATGCTGAACCACTTGTAGAC and GGTGAACCT GGATCACTCTG; Areg, TGAATCATTGCCAAGCCAC and TAAAAAGTGACAACTGGGCATC; Csn1s1, CTCCATCC ACCTCATGTCTC and CGCTCAGATGATGCAACTG; Tgfb2, ATGTCTTCAGCCGAGGTCTG and CCCACATC TTCTTTCTCTGCTC; Ccnb2, GTATTACACAGGCTAC ATGGAG and ACATACAGGATCTGAGAAGCG; Dsc2, ATGCTGTGCCTTGCTTTAG and AGCATTTGGTGT TCACAGAC; Csn1s2, TCTTCGTGGTTTCCCCATC and ACTTTAATGTCTTGGCGAGAG in 20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% Tween 20, 1 mM EGTA, 50 μM of each dNTP, and 2.5 U Taq DNA polymerase (Roche Applied Science, Indianapolis, IN). Amplification with β-actin cDNA using primers 5'-ACAGGAAGTCCCTTGCC ATC-3' and 5'-ACTGGTCTCAAGTCAGTGTACAGG-3' as the internal control was carried out by real-time PCR (iCycler, Bio-Rad) using cycle parameters 94°C for 25 sec, 55°C for 1 min, and 72°C for 1 min.

Global gene expression profiling. Total RNA was extracted from microdissected tumors (n=3 for each group) using a commercially available kit (RNEasy, Qiagen). Integrity of ribosomal RNA bands was confirmed by northern gel electrophoresis. For each hybridization, total RNA (10 μg) was converted to labeled cRNA targets. The biotinylated cRNA targets were then purified, fragmented, and hybridized to GeneChip mouse genome 430 2.0 expression arrays (Affymetrix, Santa Clara, CA) to interrogate the abundance of 39,000 possible transcripts in each sample. Affymetrix GCOS software was used to generate raw gene expression scores and normalized to the relative hybridization signal from each experiment. All gene expression scores were set to a minimum value of 2 times the background determined by GCOS software in order to minimize noise associated with less robust measurements of rare transcripts. Data was analyzed by t-test with $p<0.005$ followed by ratio analysis (minimum 2-fold change).

Results

The histopathology of NBS1+/-;neu mammary glands was consistent with that of NBS1+/+;neu animals, exhibiting normal mammary ductal structure in young mice (Fig. 1A). Mice from both genotypes developed hyperplastic terminal end buds which progressed to poorly differentiated adenocarcinoma (Fig. 1B). Monomorphic sheets of poorly differentiated epithelial cells with duct formation was observed in tumors from both NBS1+/-;neu and NBS1+/+;neu mice (Fig. 1C and D). Lung metastasis was observed in both groups of animals (Fig. 1E). The tumor latency in NBS1+/-;neu mice was dramatically increased (77 weeks vs. 40 weeks for NBS1+/+;neu animals; $p<0.01$; Fig. 2A). However NBS1+/-;neu mammary tumors were highly metastatic (4-fold increase in percentage of mice with metastatic tumors ($p<0.02$; Fig. 2B).

To understand the increased latency of NBS1+/-;neu mammary tumors, we analyzed cellular proliferation and programmed cell death. As shown in Fig. 3A, 4 week NBS1+/-;neu tumors exhibited a 7-fold increase in apoptotic cells as determined by TUNEL analysis. The percentage of apoptotic cells in eight week NBS1+/-;neu tumors was similar to that in NBS1+/+;neu mice. We also analyzed cellular proliferation in early and late stage NBS1+/-;neu and NBS1+/+;neu mammary tumors. We did not detect significant differences in the percentages of PCNA positive cells between these groups (Fig. 3B). We concluded that increased apoptosis in NBS1+/-;neu mammary tumors resulted in increased latency.

To understand the differences in mammary tumor phenotype in NBS1+/-;neu mice, we performed global gene expression profiling. Changes in cancer gene expression are shown in Tables I-III. Primary tumors from NBS1+/-;neu and NBS1+/+;neu were more highly related to each other than to metastatic tumors. Bioinformatic analysis revealed gene expression changes in specific pathways distinguishing NBS1+/-;neu from NBS1+/+;neu primary tumors, including metastasis

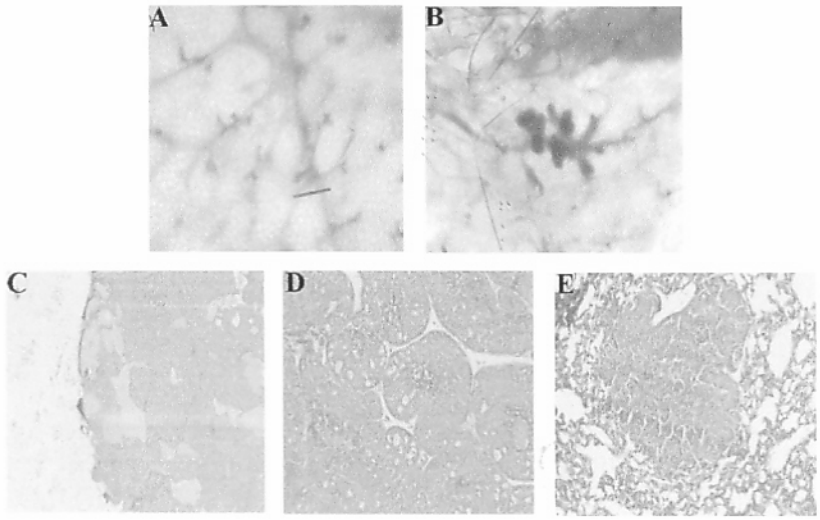

Figure 1. NBS1 haploinsufficiency increases tumor latency in the mammary tumor prone MMTV-neu mouse. (A) Whole mount preparation of mammary fat pad in NBS1+/- mouse. (B) Whole mount preparation of mammary fat pad in NBS1+/-;neu mouse showing hyperplastic terminal end buds. (C) Poorly differentiated adenocarcinoma in the mammary fat pad of NBS1+/-;neu mouse. Section is stained with hematoxylin and eosin; scale bar = 100 μm. (D) Higher magnification of poorly differentiated adenocarcinoma in NBS1+/-;neu mouse. Tumor consists of sheets of poorly differentiated epithelial cells and ducts. Scale bar = 50 μm. (E) Metastatic tumor in lung of NBS1+/-;neu mouse. Magnification is same as in (D).

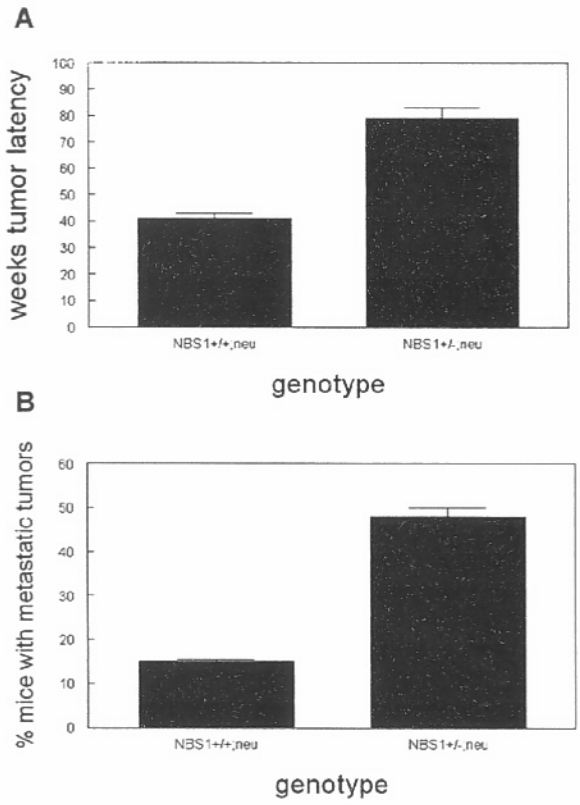

Figure 2. (A) Increased tumor latency in NBS1+/-;neu mice. The number of weeks to tumor formation in NBS1+/+;neu and NBS+/-;neu mice were determined by twice weekly palpation. (B) Increased metastatic mammary cancer in NBS1+/-;neu mice. The percentage of NBS1+/-;neu and NBS1+/+;neu mice with metastatic mammary tumors is shown. Error bars indicate SEM.

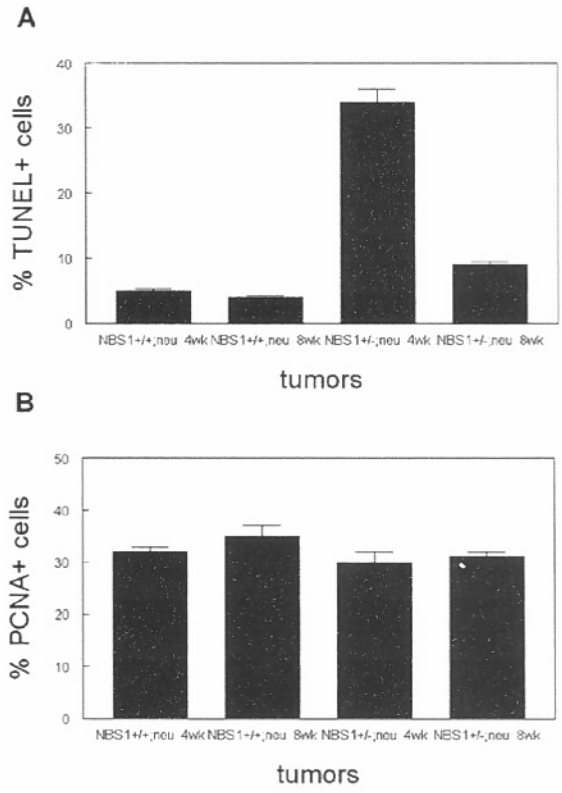

Figure 3. Increased programmed cell death in early NBS1+/-;neu mammary tumors correlates with increased latency. (A) The percentage of apoptotic cells in early and late stage NBS1+/-;neu and NBS1+/+;neu mammary tumors was determined as described in Materials and methods. (B) To assess cellular proliferation, the percentage of PCNA positive cells in early and late stage NBS1+/-;neu and NBS1+/+;neu mammary tumors was determined. Error bars indicate SEM.

Table I. Differentially expressed genes between NBS1+/+;neu and NBS+/-;neu primary mammary tumors (55 genes).

Accession	Gene name	Gene symbol	Fold change
NM_007470	Apolipoprotein D	Apod	796.0
AV325919	ATPase, Na^+/K^+ transporting, α2	Atp1a2	198.9
AF108501	Chloride channel calcium activated 2	Clca2	58.0
NM_030677	Glutathione peroxidase 2	Gpx2	54.1
AF146523	Metastasis lung adenocarcinoma 1	Malat1	43.6
NM_008183	Glutathione S-transferase, mu 2	Gstm1	31.8
NM_134032	Homeo box B2	Hoxb2	18.0
BC011063	Homeo box A5	Hoxa5	17.1
AW105779	Lactate dehydrogenase D	Ldhd	14.6
AV367068	Desert hedgehog	Dhh	13.8
NM_013605	Mucin 1, transmembrane	Muc1	12.2
AF059567	Cyclin-dependent kinase inhibitor 2B	Cdkn2b	11.4
NM_008259	Forkhead box A1	Foxa1	10.5
NM_008608	Matrix metallopeptidase 14	Mmp14	10.3
AI649186	Fibroblast growth factor 1	Fgf1	9.6
U30244	Ephrin B2	Efnb2	9.5
BE686893	Nuclear receptor coactivator 7	Ncoa7	9.0
AB049755	Mannan-binding lectin serine peptidase	Masp1	9.0
BG073383	Homeo box B3	Hoxb3	8.9
NM_008858	Protein kinase C, mu	Prkcm	8.5
BB197591	Protocadherin 7	Pcdh7	8.4
BB224034	Phospholipase C, β4	Plcb4	8.1
BB322941	Nuclear receptor subfamily 4, group A, 2	Nr4a2	7.4
NM_007556	Bone morphogenetic protein 6	Bmp6	7.0
NM_013867	Breast cancer anti-estrogen resistance 3	Bcar3	6.8
BB269715	Hypoxia inducible factor 1, α subunit	Hif1a	6.4
BB795235	Frizzled homolog 5 (*Drosophila*)	Fzd5	6.4
BB795491	DNA methyltransferase 3A	Dnmt3a	6.3
NM_007631	Cyclin D1	Ccnd1	6.3
BC024375	Growth hormone receptor	Ghr	6.2
AA214868	Phosphatase and tensin homolog	Pten	6.2
BB520860	RAR-related orphan receptor α	Rora	6.2
BB376407	Jumonji, AT rich interactive domain 1A	Jarid1a	5.9
AV359819	Jagged 1	Jag1	5.5
NM_008054	Fyn proto-oncogene	Fyn	5.4
AV241297	Serine peptidase inhibitor, Kazal type 5	Spink5	-6.4
NM_010113	Epidermal growth factor	Egf	-8.3
NM_010679	Lactalbumin, α	Lalba	-21.7
NM_009973	Casein α s2-like B	Csn1s2b	-27.2
NM_008644	Mucin 10, salivary mucin	Muc10	-100.4

(Malat1, 43.6-fold; Mmp14, 10.3-fold), cell cycle (Cdkn2b, 11.4-fold), angiogenesis (Hif1a, 6.4-fold), epidermal growth factor signaling (Egf, -8.3-fold), and differentiation (Lalba, -21.7-fold; Csn1s2b, -27.2-fold; Muc10, -100.4-fold). We also compared gene expression in NBS1+/-;neu and NBS1+/+;neu metastatic tumors. These pathways included metastasis, Notch signaling (Dlk1, 462.9-fold; Jag1, 28.6-fold; Jag2, 6.2-fold), epidermal growth factor receptor signaling (Areg, 66.3-fold; Ereg, 23.5-fold; Hbegf, 9.4-fold), fibroblast growth factor signaling (Fgfr2, 68.9-fold; Fgf1, 14.9-fold; Fgfr3, 10.9-fold), peroxisome proliferator activated receptor signaling (Pparg, 21.8-fold; Ppara, 10.1-fold, Ppargc1b, 7.1-fold), forkhead signaling (Foxa2, 42.6-fold; Foxa1, 33.6-fold; Foxp2, 19-fold; Foxq1, 18.1-fold), and differentiation (Wap, -22.1-fold; Csn3, -32.3-fold; Lalba, -212.2-fold; Csn, -310.8-fold; Csn1s1, -365.2-fold). Finally we compared gene expression in primary and metastatic NBS1+/-;neu mammary tumors. These pathways included Notch signaling (Dlk1, 308.8-fold; Jag1, 13.9-fold; Jag2, 6.2-fold), transforming growth factor/

Table II. Differentially expressed genes between NBS1+/+;neu and NBS1+/-;neu metastatic mammary tumors (239 genes).

Accession	Gene name	Gene symbol	Fold change
NM_010052	Δ-like 1 homolog (*Drosophila*)	Dlk1	462.9
NM_007663	Cadherin 16	Cdh16	140.1
NM_008471	Keratin 19	Krt19	98.6
AF146523	Metastasis lung adenocarcinoma 1	Malat1	69.8
NM_010207	Fibroblast growth factor receptor 2	Fgfr2	68.9
NM_009704	Amphiregulin	Areg	66.3
AV304616	Sonic hedgehog	Shh	44.6
NM_010446	Forkhead box A2	Foxa2	42.6
NM_007554	Bone morphogenetic protein 4	Bmp4	34.7
BF585144	Regulator of G-protein signaling 5	Rgs5	34.1
NM_008259	Forkhead box A1	Foxa1	33.6
AV022238	Chemokine (C-X-C motif) ligand 15	Cxcl15	32.7
NM_008398	Integrin α7	Itga7	30.2
AV359819	Jagged 1	Jag1	28.6
NM_007950	Epiregulin	Ereg	23.5
NM_011146	Peroxisome proliferator receptor γ	Pparg	21.8
U30244	Ephrin B2	Efnb2	21.4
NM_133721	Integrin α9	Itga9	20.7
BC002073	Chemokine (C-C motif) ligand 6	Ccl6	19.6
NM_019932	Chemokine (C-X-C motif) ligand 4	Cxcl4	19.4
NM_017399	Fatty acid binding protein 1, liver	Fabp	19.4
AV322952	Forkhead box P2	Foxp2	19.0
AV009267	Forkhead box Q1	Foxq1	18.1
AI649186	Fibroblast growth factor 1	Fgf1	14.9
NM_013867	Breast cancer anti-estrogen resistance 3	Bcar3	13.1
AV239587	Bone morphogenetic protein 2	Bmp2	11.8
NM_008010	Fibroblast growth factor receptor 3	Fgfr3	10.9
NM_008608	Matrix metallopeptidase 14	Mmp14	10.8
BB277517	Histone deacetylase 7A	Hdac7a	10.3
BC016892	Peroxisome proliferator receptor α	Ppara	10.1
BG069466	CREB binding protein	Crebbp	9.8
NM_013565	Integrin α3	Itga3	9.6
BB040443	Snail homolog 2 (*Drosophila*)	Snai2	9.5
L07264	Heparin-binding EGF-like growth factor	Hbegf	9.4
AK004683	Wingless-related MMTV integration 7A	Wnt7a	8.8
BM239177	Mitogen activated protein kinase 14	Mapk14	8.7
BC010202	Kirsten rat sarcoma viral oncogene	Kras	8.6
BB376407	Jumonji, AT rich interactive domain 1A	Jarid1a	8.2
BM119402	Sloan-Kettering viral oncogene homolog	Ski	7.7
NM_133249	Peroxisome receptor coactivator 1β	Ppargc1b	7.1
BC011118	CCAAT/enhancer binding protein α	Cebpa	6.8
NM_020265	Dickkopf homolog 2 (*Xenopus laevis*)	Dkk2	6.8
NM_008416	JunB oncogene	JunB	6.5
AV264681	Jagged 2	Jag2	6.2
BG064099	Braf transforming gene	Braf	5.8
NM_007669	Cyclin-dependent kinase inhibitor 1A	Cdkn1a	-6.1
BF144658	Transforming growth factor, β2	Tgfb2	-6.7
AU043193	Frizzled homolog 3 (*Drosophila*)	Fzd3	-6.9
BC008152	Caspase 1	Casp1	-13.3
NM_009808	Caspase 12	Casp12	-13.8
NM_011709	Whey acidic protein	Wap	-22.1
BC004601	Casein κ	Csn3	-32.3
NM_010679	Lactalbumin, α	Lalba	-212.2
NM_009972	Casein β	Csn	-310.8
NM_007784	Casein α s1	Csn1s1	-365.2

Table III. Differentially expressed genes between NBS1+/-;neu primary and metastatic tumors (222 genes).

Accession	Gene name	Gene symbol	Fold change
AV022238	Chemokine (C-X-C motif) ligand 15	Cxcl15	405.0
NM_010052	Δ-like 1 homolog (*Drosophila*)	Dlk1	308.8
BC002073	Chemokine (C-C motif) ligand 6	Ccl6	113.2
NM_008471	Keratin 19	Krt19	111.5
AV304616	Sonic hedgehog	Shh	104.9
NM_010446	Forkhead box A2	Foxa2	81.7
NM_007554	Bone morphogenetic protein 4	Bmp4	69.4
NM_008010	Fibroblast growth factor receptor 3	Fgfr3	24.8
NM_007950	Epiregulin	Ereg	23.5
NM_008404	Integrin β2	Itgb2	21.9
NM_011126	Palate, lung and nasal carcinoma	Plunc	20.1
AK007410	Growth arrest and DNA-damage 45	Gadd45g	18.3
AV009267	Forkhead box Q1	Foxq1	18.1
BC011118	CCAAT/enhancer binding protein α	Cebpa	15.4
AF416641	Hypoxia inducible factor 3, α subunit	Hif3a	15.0
AA880220	Jagged 1	Jag1	13.9
AV322952	Forkhead box P2	Foxp2	12.8
NM_010207	Fibroblast growth factor receptor 2	Fgfr2	12.3
AV311104	Cancer susceptibility candidate 4	Casc4	11.3
BM231135	Bone morphogenetic protein 1	Bmp1	11.2
NM_013565	Integrin α3	Itga3	10.3
AV032115	Bone morphogenetic protein 5	Bmp5	8.6
AF128196	Chemokine (C-C motif) ligand 9	Ccl9	8.2
NM_008261	Hepatic nuclear factor 4, α	Hnf4a	7.7
NM_011332	Chemokine (C-C motif) ligand 17	Ccl17	7.5
NM_008259	Forkhead box A1	Foxa1	7.3
BB373572	Calcium dependent protein kinase II	Camk2d	7.0
NM_020265	Dickkopf homolog 2 (*Xenopus laevis*)	Dkk2	6.8
BB486740	Hypoxia inducible factor 3, α subunit	Hif3a	6.7
BM293452	Jumonji, AT rich interactive domain 2	Jarid2	6.5
AV264681	Jagged 2	Jag2	6.2
M65143	Lysyl oxidase	Lox	6.0
NM_133654	CD34 antigen	Cd34	6.0
BC005453	V-myc viral related oncogene	Mycn	5.8
BE688115	Fibroblast growth factor 1	Fgf1	5.6
BB787243	Insulin-like growth factor binding protein	Igfbp4	5.6
BC023427	Platelet derived growth factor, B	Pdgfb	5.5
BB015508	Jumonji domain containing 1C	Jmjd1c	5.4
BB040443	Snail homolog 2 (*Drosophila*)	Snai2	5.2
BQ175880	Cyclin D2	Ccnd2	5.0
BB543291	Chemokine (C-C motif) receptor-like 1	Ccrl1	5.0
NM_011121	Polo-like kinase 1 (*Drosophila*)	Plk1	-5.4
BC027242	Vav 3 oncogene	Vav3	-5.8
X75483	Cyclin A2	Ccna2	-5.8
BC003261	Aurora kinase B	Aurkb	-6.8
NM_009525	Wingless-related integration site 5B	Wnt5b	-6.9
BF144658	Transforming growth factor, β2	Tgfb2	-8.4
NM_019645	Plakophilin 1	Pkp1	-8.4
AK013312	Cyclin B2	Ccnb2	-8.4
AV367068	Desert hedgehog	Dhh	-8.8
AU043193	Frizzled homolog 3 (*Drosophila*)	Fzd3	-10.5
NM_013505	Desmocollin 2	Dsc2	-11.9
U03425	Epidermal growth factor receptor	Egfr	-18.5
BC014690	Transforming growth factor, β3	Tgfb3	-29.2
NM_007785	Casein α s2-like A	Csn1s2a	-127.4
NM_009972	Casein β	Csn	-160.8
NM_007786	Casein κ	Csn3	-958.5

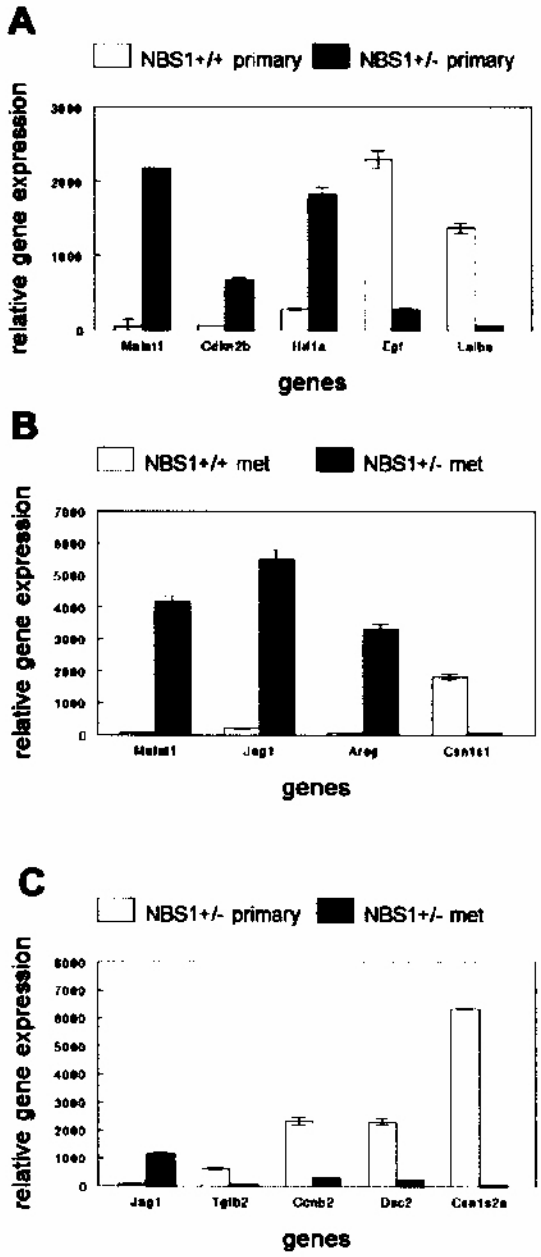

Figure 4. NBS1+/-;neu mammary tumors exhibit specific gene expression signatures. (A) Relative mRNA expression of genes in NBS1+/+;neu and NBS1+/-;neu primary mammary tumors was performed by qRT-PCR. (B) Relative mRNA expression of genes in NBS1+/+;neu and NBS1+/-;neu metastatic mammary tumors was performed by qRT-PCR. (C) Relative mRNA expression of genes in NBS1+/-;neu primary and metastatic mammary tumors was performed by qRT-PCR. Error bars indicate SEM.

bone morphogenetic protein signaling (Bmp4, 69.4-fold; Bmp1, 11.2-fold; Bmp5, 8.6-fold; Tgfb2, -8.4-fold), cell cycle (Ccnd2, 5-fold; Ccna2, -5.8-fold; Ccnb2, -8.4-fold), cell adhesion (Pkp1, -8.4-fold; Dsc2, -11.9-fold), forkhead signaling (Foxa2, 81.7-fold; Foxq1, 18.1-fold; Foxp2, 12.8-fold), epidermal growth factor signaling (Ereg, 23.5-fold; Egfr, -18.5-fold), and differentiation (Csn1s2a, -127.4-fold; Csn -160.8-fold; Csn3, -958.5-fold). Expression of representative genes involved in these pathways was validated by qRT-PCR in Fig. 4. These results demonstrated specific patterns of gene expression that correlated with primary and metastatic tumor phenotype.

Discussion

Mutations in the NBS1 gene have been associated with increased risk of breast cancer (14-16). Persistent radiation induced NBS1 foci has been associated with chromosomal instability and increased breast cancer risk (13). In mice, NBS1 null mutation is embryonic lethal but heterozygosity renders mice susceptible to tumor formation (5). However, mammary tumors are uncommon in mouse strains with reduced NBS1 function (6). To examine the role of NBS1 in mammary tumor formation, we examined NBS1 haploinsufficiency in the mammary tumor prone MMTV-neu strain. Reduced expression of NBS1 resulted in increased apoptosis in NBS1 heterozygous mice. This increased cell death correlated with markedly increased tumor latency in NBS1 heterozygous mice. These effects were likely due to decreased DNA repair following oncogene induced cellular proliferation. Defects in cellular proliferation were noted in the cells of NBS1 deficient mice in previous studies (6). Increased latency correlated with decreased growth factor expression and increased cyclin dependent kinase inhibitor expression. Loss of p53 has been shown to greatly increase tumorigenesis in NBS1 mutant mice, suggesting that p53 mediated DNA damage response may be responsible for apoptosis and increased tumor latency (15). A previous study demonstrated nuclear export of NBS1 following ionizing radiation as a mechanism of downregulating the DNA damage response (16). Previous studies have demonstrated increased chromosomal aberrations in NBS1+/- tumors (5). Loss of NBS1 has been shown to induce supernumerary centrosomes similar to those observed in BRCA1 deficient cells, leading to increased chromosomal instability (17). These studies demonstrate that impaired NBS1 function can result in cellular proliferation defects leading to increased tumor latency. It is interesting to speculate that tumorigenic clones that escape defective cell death pathways may be more aggressive and metastatic due to chromosomal aberrations induced by diminished NBS1 function. In support this hypothesis, increased numbers of differentially expressed genes and degregulated signaling pathways in NBS1+/-;neu tumors correlated with increased metastatic disease.

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Regulation of the Nijmegen breakage syndrome 1 gene NBS1 by c-myc, p53 and coactivators mediates estrogen protection from DNA damage in breast cancer cells

ROWENA WAN, JIANCHUN WU, KAITRIN K. BALOUE and DAVID L. CROWE

University of Illinois Cancer Center, Chicago, IL 60612, USA

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Abstract. In mammalian cells more than 90% of double-strand breaks are repaired by NHEJ. Impairment of this pathway is associated with cell cycle arrest, cell death, genomic instability and cancer. Human diseases such as Nijmegen breakage syndrome, due to mutations in the NBS1 gene, produce defects in resection of double-strand breaks. NBS1 hypomorphic mutant mice are viable, and cells from these mice are defective in S phase and G2/M checkpoints. NBS1 polymorphisms have been associated with increased risk of breast cancer. We previously demonstrated that estradiol protected estrogen receptor (ER)-positive (+) breast cancer cell lines against double-strand breaks and cell death. We now demonstrate that protection from double-strand break damage in ER$^+$ cells is mediated via regulation by c-myc, p53, CBP and SRC1 coactivators in intron 1 of the NBS1 gene. We concluded that NBS1 is responsible for estradiol-mediated protection from double-strand breaks in ER$^+$ breast cancer cells.

Introduction

Double-strand break repair is mediated by two major repair pathways, homologous recombination (HR) or non-homologous end joining (NHEJ; reviewed in ref. 1). In mammalian cells more than 90% of double-strand breaks are repaired by NHEJ. Impairment of either pathway is associated with cell cycle arrest, cell death, genomic instability and cancer (2). Human diseases such as Nijmegen breakage syndrome (NBS) due to mutations in the NBS1 gene result in defects in resection of double-strand breaks (3). NBS1 functions as part of the Mre11/Rad50/NBS1 (MRN) complex whose functions are not restricted to HR but are also involved NHEJ (4).

NBS is a rare human autosomal recessive disorder caused by hypomorphic mutations. This disorder is characterized by growth retardation, immunodeficiency, microcephaly and cancer predisposition. At the cellular level, NBS is characterized by radiosensitivity, chromosomal breakage and defective cell cycle checkpoints. NBS1 null mutations result in early embryonic lethality (5), but NBS1 hypomorphic mutants are viable (6). Cells from these mice are defective in S phase and G2/M checkpoints. Heterozygous mice with an NBS1 null mutation in addition to homozygous animals with hypomorphic mutations are predisposed to cancer. Conditional NBS1 mutant mice have been characterized (7). For example, neuronal inactivation of NBS1 results in chromosomal breaks, microcephaly, growth retardation, cerebellar defects and ataxia. The MRN complex is essential for maintaining genomic integrity, cell viability and checkpoint activation.

MRN polymorphisms have been associated with increased risk of breast cancer (8-10). MRN expression was reduced in the majority of breast tumors (11). Low expression of MRN correlated with increased histologic grade and estrogen receptor negativity. Response to radiotherapy correlated with high expression of the MRN complex. Patients with high numbers of ionizing radiation induced NBS1 foci had aggressive breast cancer phenotypes (12,13).

Estradiol has been shown to markedly enhance proliferation of mammary gland epithelium and estrogen receptor (ER) α positive (+) breast cancer cells (14). ER is a member of a large family of ligand dependent transcription factors that include steroid, retinoid, thyroid and vitamin D receptors. ER have functional domains for DNA binding, ligand binding, dimerization, and transcriptional activation. Nuclear receptors such as ER require coactivator proteins such as CREB binding protein (CBP) and steroid receptor coactivator 1 (SRC1) to activate target gene transcription (15). We previously demonstrated that estradiol protected ER$^+$ breast cancer cell lines against double-strand breaks and cell death (16). Ectopic ER expression was sufficient to produce these effects and this protection involved the coactivator CBP. We now demonstrate that this protection from double-strand break damage is mediated via regulation by c-myc, p53 and coactivators in intron 1 of the NBS1 gene.

Correspondence to: Professor David L. Crowe, University of Illinois Cancer Center, 801 S. Paulina Street, Room 530C, Chicago, IL 60612, USA
E-mail: dlcrowe@uic.edu

Key words: Rad50, Mre11, MMTV-neu, p53, transcription factor

Materials and methods

Cell culture and stable transfection. The human mammary epithelial and breast cancer cell lines used in this study were purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium without phenol red, 10% charcoal-resin treated fetal bovine serum, and 40 μg/ml gentamicin in a humidified atmosphere of 5% CO_2 at 37°C. Cultures were treated with 100 nM E2 for 4 h, 3 Gy ionizing radiation, combined E2 and radiation or vehicle. For some experiments, cells were transfected with 2 μg c-myc, CBP, SRC1 or neomycin resistance plasmid using Lipofectamine according to manufacturer's recommendations (Invitrogen, Carlsbad, CA). Cells were selected in 400 μg/ml G418 for 14 days. Resistant clones were picked for expansion and characterization. For inhibition of gene expression experiments, cells were transfected with siRNA to ERα, Mre11, Rad50, NBS1 or control siRNA according to manufacturer's protocol (Dharmacon, Lafayette, CO).

DNA damage and apoptosis analysis. DNA damage was quantitated by single cell gel electrophoresis. Treated cells were mixed with 0.5% low melting point agarose and added to microscope slides coated with 1.5% agarose. Cells were alkali denatured (pH 13.0), subjected to electrophoresis at 0.86 V/cm for 25 min and stained with ethidium bromide. The tail moment (DNA migration x tail intensity) of 50 randomly selected cells was analyzed from each slide using imaging software. For apoptosis assays, human mammary epithelial and breast cancer cell cultures were fixed with 70% ethanol at -20°C for 30 min and washed with PBS. Cultures of mouse mammary epithelial cells were incubated with terminal deoxynucleotidyl transferase and fluorescein conjugated dUTP at 37°C for 30 min followed by washing in PBS. The percentage of apoptotic cells was determined by flow cytometry.

Western blot analysis. Protein was extracted in 1X Laemmli buffer from treated human mammary epithelial and breast cancer cell lines. Total cellular protein (75 μg) was separated by SDS-PAGE on 10% resolving gels under denaturing and reducing conditions. Separated proteins were electroblotted to PVDF membranes according to manufacturer's recommendations (Roche Applied Science, Indianapolis, IN). Blots were incubated with antibodies to ERα, Mre11, Rad50, NBS1, c-myc, CBP, SRC1 or β-actin for 16 h at 4°C. After washing in Tris buffered saline containing 0.1% Tween-20 (TBST, pH 7.4), blots were incubated for 30 min at room temperature with anti-IgG secondary antibody conjugated to horseradish peroxidase. Following extensive washing in TBST, bands were visualized by the enhanced chemiluminescence method (Roche Applied Science). Bands were quantitated by laser densitometry.

Chromatin immunoprecipitation. Treated human mammary epithelial or breast cancer cells were fixed in 1% formaldehyde for 10 min at room temperature. Cells were washed in PBS and lysed in immunoprecipitation buffer containing protease inhibitors for 30 min at 4°C, sheared and centrifuged at 10,000 x g for 10 min. Supernatants were cleared with 2 μg sheared salmon sperm DNA, 20 μl preimmune serum, and 20 μl protein A/G sepharose beads for 2 h at 4°C. Aliquots of the supernatant were used as input DNA for normalization. Immunoprecipitation using anti-myc, -p53, -CBP, -SRC1 or -acetylated histone H3 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) was performed overnight at 4°C. Preimmune IgG was used as the negative control antibody. Immunoprecipitates were washed extensively in immunoprecipitation buffer, resuspended in 10 mM Tris-HCl, 1 mM EDTA (TE, pH 8.0) and incubated at 65°C for 6 h to reverse crosslinks. The supernatants were extracted with phenol/chloroform and ethanol precipitated. Following washing in 70% ethanol, pellets were dried and suspended in 50 μl TE. For real-time PCR, 1 μl of template was amplified in buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 nM each dNTP and 100 ng each primer (5'-GATAACCCTTTCCCACTGATTG-3' and 5'-GAGA ACTGCTTGAACCCAG-3') flanking the myc and p53 binding sites in the NBS1 first intron (accession AY566246; 3024-3029 bp and 3124-3134 bp, respectively). The optimized cycle parameters were one cycle at 94°C for 3 min followed by 25 cycles of 94°C for 25 sec, 58°C for 60 sec, 72°C for 60 sec and one final cycle at 72°C for 10 min (iCycler, Bio-Rad).

Nucleosomal mapping. Nuclei were isolated from parental human breast cancer cell lines treated with E2, IR, E2+IR, or vehicle. Chromatin was digested to mononucleosomal form with micrococcal nuclease (Roche Applied Science). The digestion was stopped by addition of 50 mM EDTA. Nuclei were lysed in 1% SDS and treated with 0.1 mg proteinase K overnight at 37°C. DNA was purified by phenol/chloroform extraction and ethanol precipitation. DNA was suspended in TE buffer and analyzed by agarose gel electrophoresis to ensure digestion to mononucleosomal fragments. These fragments were eluted from the gel and used as PCR templates to determine nucleosomal occupancy of NBS1 intron 1. Undigested genomic DNA was used as the positive control and template free samples were used as the negative control.

Transient transfection, double-strand break repair and NBS1 intron 1 analysis. pACT-luc luciferase vector was digested with *Xba*I and *Bst*EII or *Eco*RV and *Xho*I restriction enzymes and purified by gel electrophoresis followed by end-filling with Klenow fragment of DNA polymerase (17). A total of 1 μg of each linear plasmid was transiently transfected into triplicate cultures of 50% confluent human breast cancer cell lines using Lipofectamine according to the manufacturer's recommendations (Invitrogen). The 728 bp homology between the two plasmids can reconstitute luciferase activity which correlates with DNA double-strand break repair activity. Undigested pACT-luc vector was used as the positive control and 1 μg β-galactosidase expression plasmid was used to normalize for transfection efficiency. In separate experiments, triplicate cultures of 50% confluent cells were transiently transfected with 2 μg of pGL3 luciferase reporter vector containing 5' flanking constructs of the NBS1 promoter, exon 1 and intron 1 (-360/+1076), lacking the promoter (-17/+1076) or lacking intron 1 (-360/+88). Intron 1 was cloned into pGL3 vector and transiently transfected with 1 μg c-myc, p53, CBP, SRC1

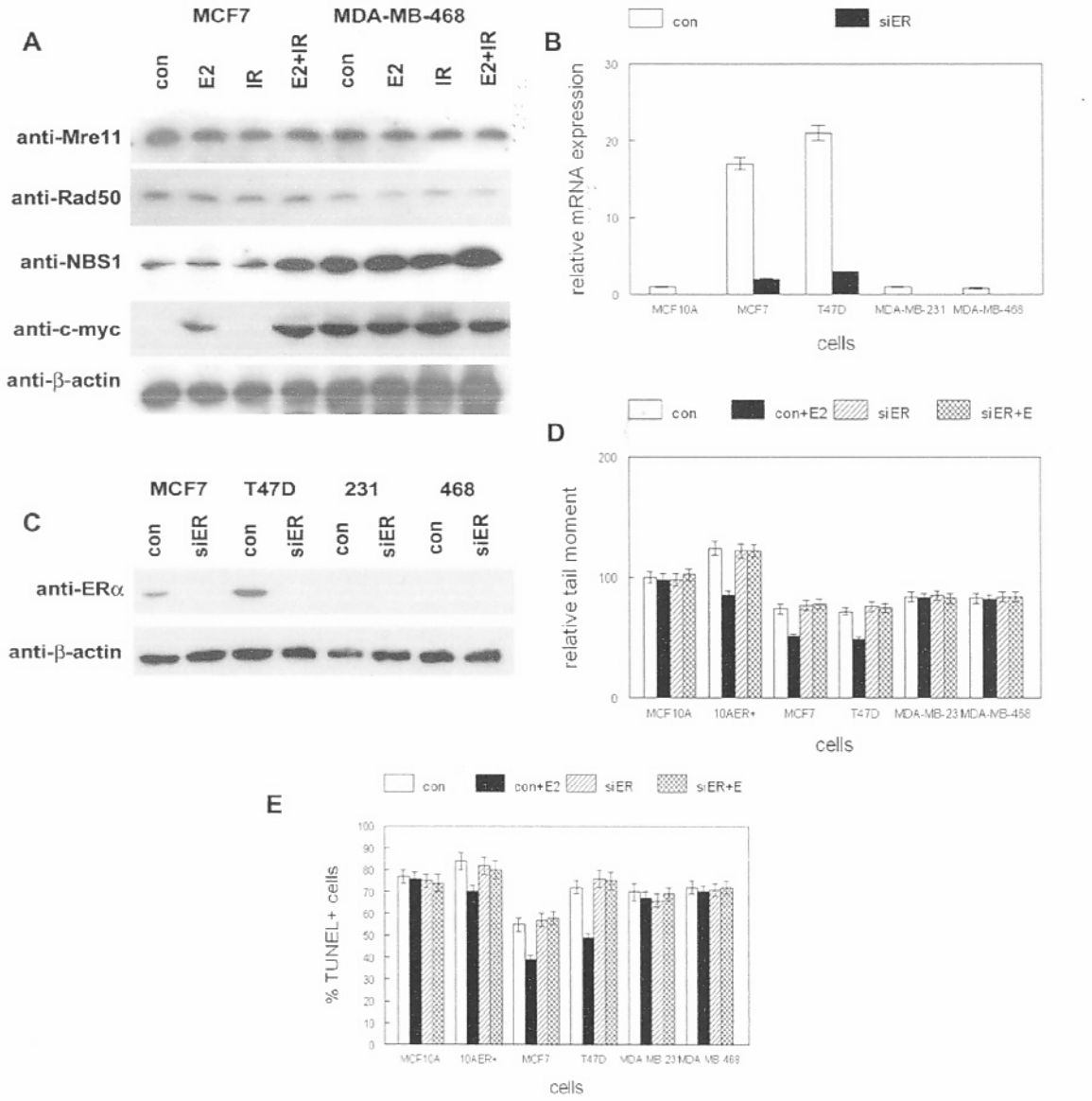

Figure 1. The combination of estradiol and ionizing radiation induces NBS1 expression in ER+ human breast cancer cell lines. (A) The ER+ breast cancer cell line MCF7 and ER- line MDA-MB-468 were treated with estradiol (E2), ionizing radiation (IR), combination (E2+IR) or vehicle (con). Protein extracts from these cells were subjected to western blot analysis using antibodies indicated at left. (B) Human breast cancer cell lines were transfected with ER or control (con) siRNA followed by qRT-PCR. (C) Human breast cancer cell lines were transfected with ER or control (con) siRNA followed by western blot analysis using antibodies indicated at left. (D) E2 protection from ionizing radiation induced DNA damage is dependent on ER expression. The immortalized human breast epithelial line MCF10A, MCF10A stably expressing ER (10AER+), and human breast cancer cell lines were transfected with ER siRNA (siER) or control siRNA (con), treated with E2 (+E2) or vehicle, and exposed to ionizing radiation. Relative tail moment is shown. (E) E2 protection from ionizing radiation induced apoptosis is dependent on ER expression. MCF10A, MCF10A stably expressing ER (10AER+) and human breast cancer cell lines were transfected with ER siRNA (siER) or control siRNA (con), treated with E2 (+E2) or vehicle, and exposed to ionizing radiation. Percent TUNEL positive cells is shown. Error bars represent SEM of three independent experiments.

or blank expression plasmids. Separate cultures were transfected with pGL3 vector containing point mutations in the intron 1 myc (CACcaGC) or p53 (GGGgccGCTCC) binding sites. Cultures were treated with E2, ionizing radiation or vehicle for 24 h. Cells were harvested and reporter gene activity determined using a commercially available kit and luminometer (Applied Biosystems, Carlsbad, CA). Luciferase activity was normalized to β-galactosidase levels for each sample. Statistical significance was determined by ANOVA.

Results

We previously determined that estradiol (E2) treatment decreased DNA damage and increased survival of ER+ human breast cancer cell lines exposed to ionizing radiation (IR) (16). To determine the mechanism of this protection, we treated ER+ and ER- human breast cancer cell lines with E2, IR or E2 followed by IR. As shown in Fig. 1A, NBS1 protein expression was induced by 5-fold in ER+ MCF7 cells when treated

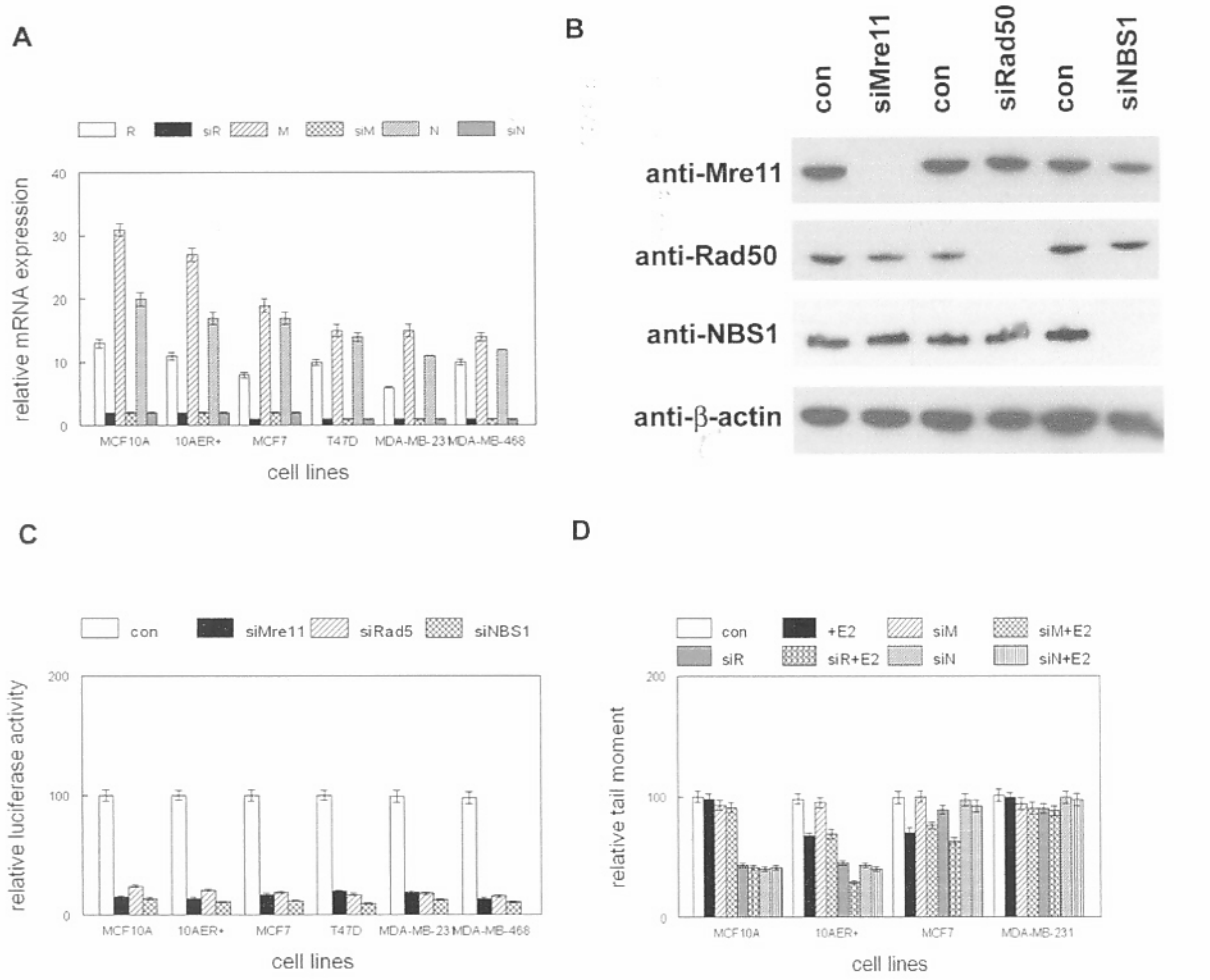

Figure 2. siRNA inhibits expression of MRN gene products. (A) MCF10A, MCF10A stably expressing ER (10AER$^+$), and human breast cancer cell lines were transfected with siRNA to Mre11 (siM), Rad50 (siR), NBS1 (siN) or control siRNAs (M, R, N) followed by qRT-PCR. (B) MCF10A, MCF10A stably expressing ER (10AER$^+$), and human breast cancer cell lines were transfected with siRNA to Mre11 (siMre11), Rad50 (siRad50), NBS1 (siNBS1) or control siRNA (con) followed by western blot analysis with antibodies indicated at left. (C) siRNAs to MRN gene products inhibits double-strand break repair. MCF10A, MCF10A stably expressing ER (10AER$^+$), and human breast cancer cell lines were transfected with siRNA to Mre11 (siMre11), Rad50 (siRad50), NBS1 (siNBS1) or control siRNA (con) and digested luciferase reporter vector. (D) NBS1 mediates E2 protection from IR mediated DNA damage. MCF10A, MCF10A stably expressing ER (10AER$^+$), and human breast cancer cell lines were transfected with siRNA to Mre11 (siM), Rad50 (siK), NBS1 (siN) or control siRNA (con), treated with E2 (+E2) or vehicle, and exposed to ionizing radiation. Relative tail moment is shown. (E) NBS1 mediates E2 protection from IR mediated apoptotic cell death. MCF10A, MCF10A stably expressing ER (10AER$^+$), and human breast cancer cell lines were transfected with siRNA to Mre11 (siM), Rad50 (siR), NBS1 (siN) or control siRNA (con), treated with E2 (+E2) or vehicle, and exposed to ionizing radiation. Percent of apoptotic cells is shown. Error bars indicate SEM of three independent experiments.

with E2 followed by IR. No NBS1 expression changes were observed in ER$^-$ MDA-MB-468 cells. No changes in expression of other MRN gene products (Mre11, Rad50) in response to E2 or IR were observed. Similar results were observed in ER$^+$ T47D and ER$^-$ MDA-MB-231 cells (data not shown). These results indicate that both E2 and IR were required to induce NBS1 expression in ER$^+$ breast cancer cell lines.

To determine if E2 mediated protection from double-strand break damage was dependent on ER, we transfected ER$^+$ and ER$^-$ human mammary epithelial and breast

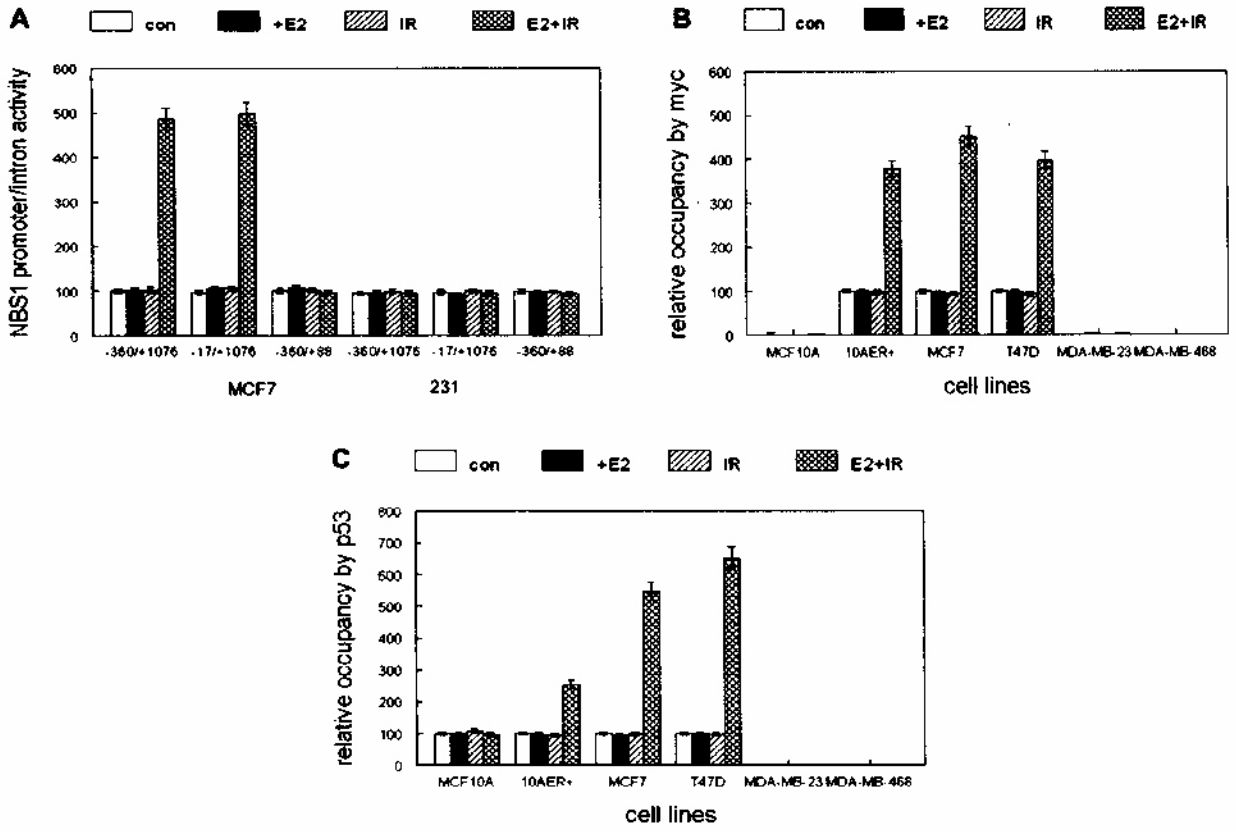

Figure 3. (A) Intron 1 mediates E2 and IR induction of NBS1 gene expression. Constructs containing the NBS1 promoter, exon 1 and intron 1 (-360/+1076), lacking the promoter (-17/+1076) or lacking intron 1 (-360/+88) were fused to the luciferase reporter and transiently transfected into MCF7 or MDA-MB-231 cells. (B) c-myc occupancy of NBS1 intron 1 is induced by the combination of E2 and ionizing radiation in ER$^+$ cells. MCF10A, MCF10A stably expressing ER (10AER$^+$), and human breast cancer cell lines were treated with E2 (+E2), ionizing radiation (IR), combined E2 and IR (E2+IR), or vehicle (con) and subjected to chromatin immunoprecipitation. Relative occupancy of intron 1 by c-myc is shown. (C) p53 occupancy of NBS1 intron 1 is induced by the combination of E2 and ionizing radiation in ER$^+$ cells. MCF10A, MCF10A stably expressing ER (10AER$^+$), and human breast cancer cell lines were treated with E2 (+E2), ionizing radiation (IR), combined E2 and IR (E2+IR), or vehicle (con) and subjected to chromatin immunoprecipitation. Relative occupancy of intron 1 by p53 is shown.

cancer cell lines with siRNA to ER. Expression of ER mRNA and protein following siRNA transfection is shown in Fig. 1B and C. ER expression was reduced in ER$^+$ MCF7 and T47D cells by >90% following siRNA transfection. Treatment of ER$^+$ cells with E2 reduced double-strand break damage by 25-30% following IR as determined by tail moment ($p<0.04$; Fig. 1D). This protective effect was abolished by ER siRNA transfection and was not observed in ER$^-$ cells. Treatment of ER$^+$ cells with E2 reduced apoptosis by similar magnitude as determined by TUNEL assay ($p<0.03$; Fig. 1E). This effect was completely inhibited by ER siRNA transfection and was not observed in ER$^-$ cells. These results indicate that the protective effect of E2 against double-strand break damage was mediated by ER.

Pretreatment with E2 prior to ionizing radiation induced NBS1 expression in ER$^+$ human mammary epithelial and breast cancer cells (Fig. 1). To determine the role of the MRN complex in mediating this response, we transfected these cells with siRNAs to Mre11, Rad50 or NBS1. Expression of these gene products following siRNA transfection is shown in Fig. 2A and B. Expression of Mre11, Rad50 or NBS1 was reduced by 90% in siRNA transfected cultures. Transfection of siRNAs to Mre11, Rad50, or NBS1 inhibited recombination of luciferase plasmids by 70-80% ($p<0.002$; Fig. 2C), providing functional confirmation of reduced expression of these gene products. To determine the effects of MRN gene product inhibition, we exposed siRNA transfected cells to E2 or vehicle followed by IR. As shown in Fig. 2D, NBS1 siRNA blocked the protective effects of E2 against double-strand break damage. These effects were observed only in ER$^+$ cells. Similar effects of NBS1 siRNA were observed on E2 mediated protection against ionizing radiation induced apoptosis (Fig. 2E). These results indicate that NBS1 mediates the E2 protective effects against ionizing radiation induced double-strand break damage and apoptosis.

To determine if NBS1 induction was mediated by transcription, we transiently transfected a reporter construct containing the 5' flanking, exon 1 and intron 1 regions of the gene into human breast cancer cell lines prior to E2 and IR treatment. As shown in Fig. 3A, the combination of E2 and IR induced reporter activity by 5-fold in ER$^+$ MCF7 cells ($p<0.01$). Deletion of the proximal promoter region had no effect on reporter activity, but removal of intron 1 completely abolished luciferase expression. These effects were not

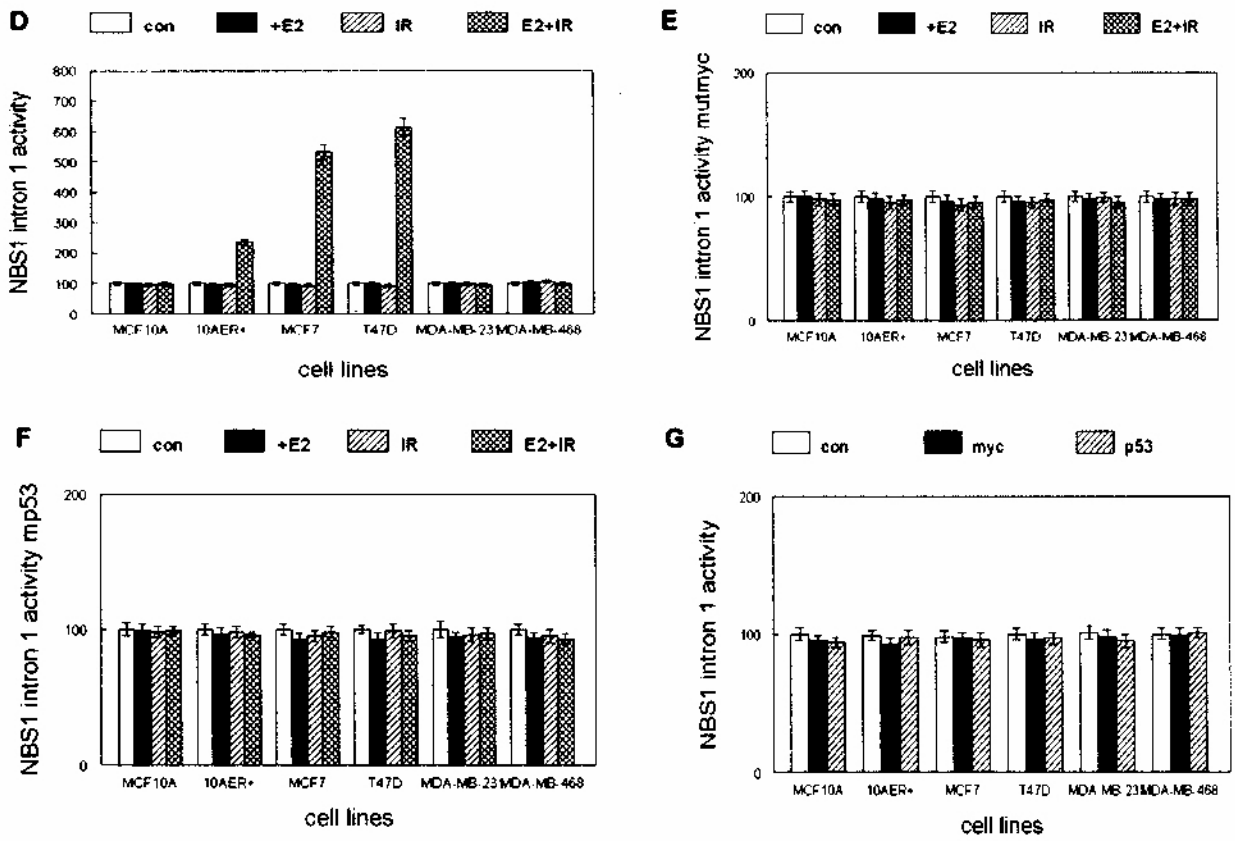

Figure 3. Continued. (D) NBS1 intron activity is induced by the combination of E2 and ionizing radiation in ER+ cells. MCF10A, MCF10A stably expressing ER (10AER+), and human breast cancer cell lines were transfected with the luciferase reporter construct and treated with E2 (+E2), ionizing radiation (IR), combined E2 and IR (E2+IR) or vehicle (con). Relative luciferase activity is shown. (E) Mutation of the E box site in the NBS1 intron 1 inhibits induction of activity by the combination of E2 and IR. MCF10A, MCF10A stably expressing ER (10AER+), and human breast cancer cell lines were transfected with the luciferase reporter construct containing a mutation in the E box site and treated with E2 (+E2), ionizing radiation (IR), combined E2 and IR (E2+IR) or vehicle (con). Relative luciferase activity is shown. (F) Mutation of the p53 binding site in the NBS1 intron 1 inhibits induction of activity by the combination of E2 and IR. MCF10A, MCF10A stably expressing ER (10AER+), and human breast cancer cell lines were transfected with luciferase reporter construct containing a mutation in the p53 binding site and treated with E2 (+E2), ionizing radiation (IR), combined E2 and IR (E2+IR) or vehicle (con). Relative luciferase activity is shown. (G) c-myc and p53 individually fail to activate NBS1 intron 1. MCF10A, MCF10A stably expressing ER (10AER+), and human breast cancer cell lines were transfected with the luciferase reporter construct and expression vectors for c-myc, p53 or control vector (con). Error bars indicate SEM of three independent experiments.

observed in ER- MDA-MB-231 cells. *In silico* analysis of potential transcription factor binding sites in NBS1 intron 1 mediating E2 and DNA damage responses revealed a myc and p53 site in close proximity. To determine relative occupancy of the myc and p53 binding sites in the NBS1 intron 1 in response to E2 and IR we performed chromatin immunoprecipitation. As shown in Fig. 3B, the combination of E2 and IR enhanced binding of c-myc to this region of NBS1 intron 1 by 4-fold in ER+ human breast epithelial cell lines ($p<0.02$). E2 or IR alone had no effect on c-myc binding to the NBS1 intron 1. ER- cells showed no binding of c-myc to the NBS1 intron 1. Similarly the combination of E2 and IR induced p53 binding to this region by 4-7-fold in ER+ cells ($p<0.01$; Fig. 3C). E2 or IR alone had no effect on p53 binding to NBS1 intron 1. ER- cells showed no binding of p53 to the NBS1 intron 1. NBS1 intron 1 activity was strongly induced by the combination of E2 and IR (2-6-fold in ER+ cells; $p<0.05$; Fig. 3D). E2 or IR alone had no effect on NBS1 intron 1 activity and no induction was observed in ER- cells. Mutation of the myc or p53 binding sites abolished the inductive effects of E2 and IR on NBS1 intron 1 activity (Fig. 3E and F). Transient overexpression of c-myc or p53 alone failed to activate NBS1 intron 1 activity (Fig. 3G). These results indicate that E2 and IR were required to activate the NBS1 intron 1 via cooperative c-myc and p53 binding to their cognate binding sites.

We previously determined that the protective effects of E2 on IR induced DNA damage was dependent on the epigenetic coactivator CBP (16). To determine if the coactivators CBP and SRC1 were recruited to the myc and p53 binding sites of the NBS1 intron 1, we performed chromatin immunoprecipitation. As shown in Fig. 4A and B, the combination of E2 and IR recruited CBP and SRC1 to this region of intron 1 in ER+ cells (2-3-fold increased occupancy; $p<0.04$). Corresponding acetylation of histone H3 in this region was increased by 2-3-fold which reduced nucleosomal occu-

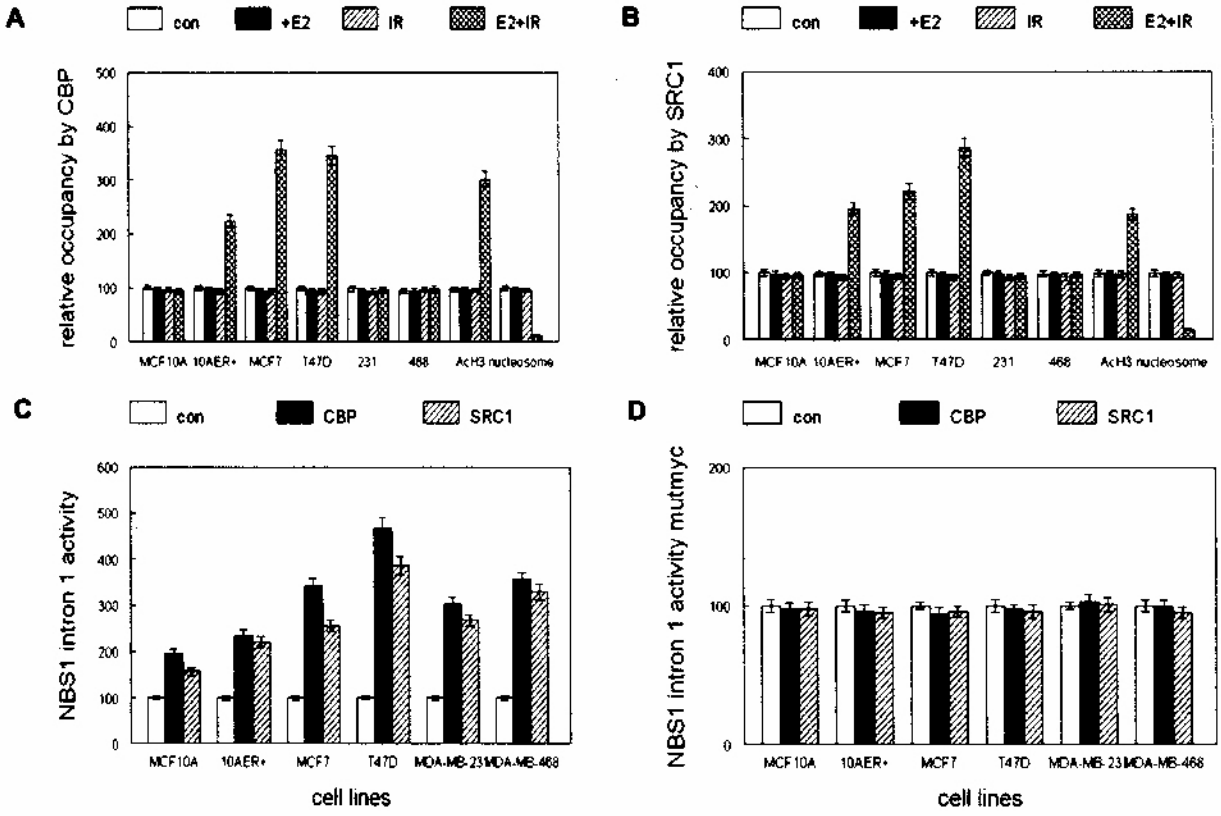

Figure 4. CBP and SRC1 coactivators are recruited to the NBS1 intron 1 by the combination of E2 and ionizing radiation. (A) MCF10A, MCF10A stably expressing ER (10AER+), and human breast cancer cell lines were treated with E2 (+E2), ionizing radiation (IR), combined E2 and IR (E2+IR) or vehicle (con) and subjected to chromatin immunoprecipitation. Relative occupancy of intron 1 by CBP, AcH3, or nucleosomes is shown. (B) MCF10A, MCF10A stably expressing ER (10AER+), and human breast cancer cell lines were treated with E2 (+E2), ionizing radiation (IR), combined E2 and IR (E2+IR) or vehicle (con) and subjected to chromatin immunoprecipitation. Relative occupancy of intron 1 by SRC1, AcH3, or nucleosomes is shown. (C) CBP and SRC1 activate NBS1 intron 1 activity. MCF10A, MCF10A stably expressing ER (10AER+) and human breast cancer cell lines were transiently transfected with luciferase reporter construct and expression vectors for CBP, SRC1 or control vector (con). (D) Mutation of the E box site inhibits coactivator mediated induction of NBS1 intron 1 activity. MCF10A, MCF10A stably expressing ER (10AER+) and human breast cancer cell lines were transfected with luciferase reporter construct containing a mutation in the E box site and expression vectors for CBP, SRC1 or control vector (con).

pancy by 90%. E2 or IR alone did not increase coactivator occupancy of this region and no increase in CBP or SRC1 binding was observed following E2 and IR treatment of ER- cells. However, transient overexpression of CBP induced NBS1 intron 1 activity by 2-5-fold and SRC1 overexpression induced intron activity by 2-4-fold in both ER+ and ER- cell lines ($p<0.01$; Fig. 4C). Mutation of the myc or p53 binding sites abolished the ability of CBP or SRC1 to induce NBS1 intron 1 activity (Fig. 4D and E). Constitutive overexpression of p53 induced apoptosis in human breast cancer cell lines (data not shown), but stable c-myc expression in combination with IR induced endogenous NBS1 expression by 3-5-fold in ER+ cell lines (Fig. 4F). CBP or SRC1 stable overexpression was sufficient to induce NBS1 gene expression by 2-4-fold in ER+ human breast cancer cell lines (Fig. 4G). These results indicate that E2 and IR recruited coactivators to the myc and p53 binding sites of the NBS1 intron 1. Coactivator induction of NBS1 gene expression was dependent on these sites, and c-myc functionally substituted for E2 treatment in ER+ cells. CBP and SRC1 functionally substituted for E2 and IR induction of NBS1 gene expression, indicating that the coactivators were sufficient to reproduce these effects.

Discussion

Our previously published studies indicated that E2 treatment decreased DNA damage and improved survival of ER+ human breast cancer cell lines following IR treatment (16). We now demonstrate that the combination of E2 and IR treatment induces NBS1 expression in ER+ but not ER- human breast cancer cell lines. While inhibition of gene products in the MRN complex inhibited DNA repair, NBS1 was responsible for mediating the anti-apoptotic effects of E2 in irradiated ER+ breast cancer cell lines. A previous study demonstrated that cells from mice expressing a C-terminal deleted NBS1 exhibited decreased apoptosis (18). Additionally E2 was previously shown to sustain the growth of irradiated breast cancer cell lines (19). This effect was due to inactivation

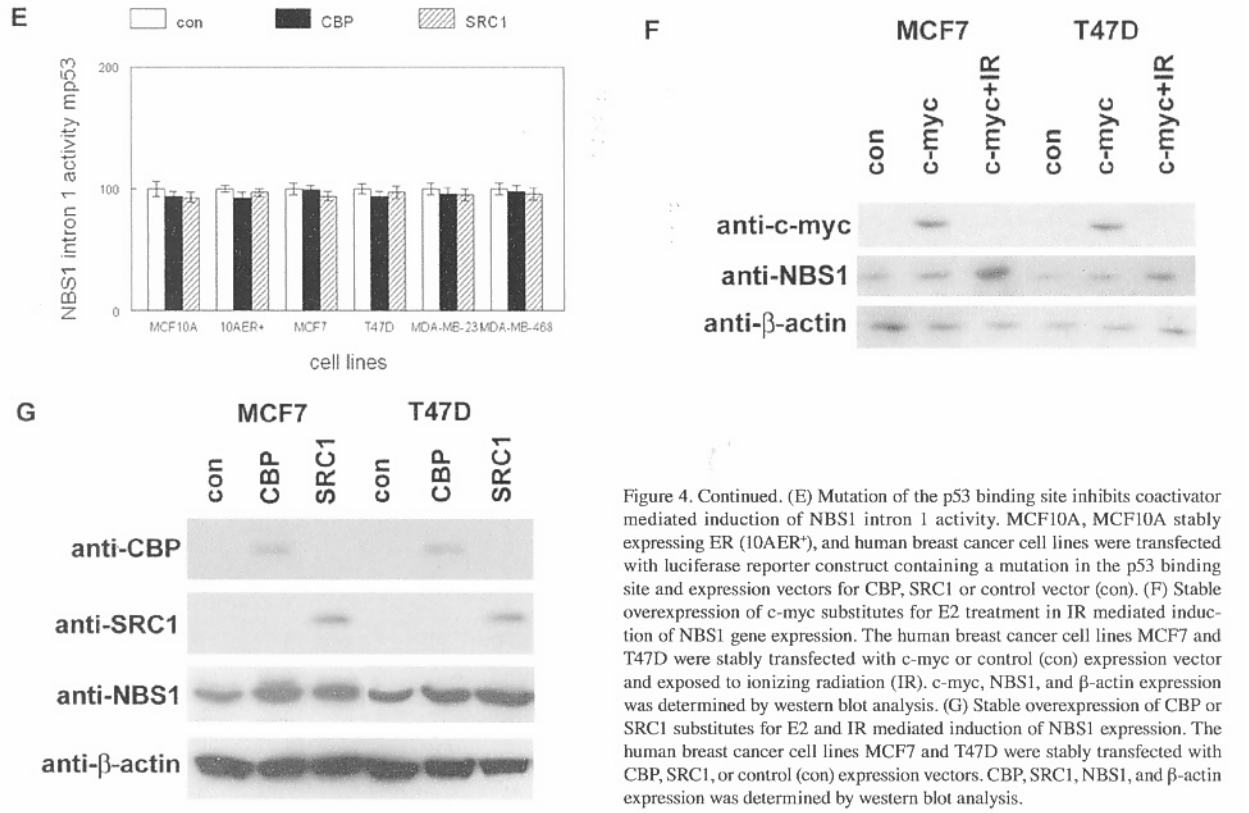

Figure 4. Continued. (E) Mutation of the p53 binding site inhibits coactivator mediated induction of NBS1 intron 1 activity. MCF10A, MCF10A stably expressing ER (10AER+), and human breast cancer cell lines were transfected with luciferase reporter construct containing a mutation in the p53 binding site and expression vectors for CBP, SRC1 or control vector (con). (F) Stable overexpression of c-myc substitutes for E2 treatment in IR mediated induction of NBS1 gene expression. The human breast cancer cell lines MCF7 and T47D were stably transfected with c-myc or control (con) expression vector and exposed to ionizing radiation (IR). c-myc, NBS1, and β-actin expression was determined by western blot analysis. (G) Stable overexpression of CBP or SRC1 substitutes for E2 and IR mediated induction of NBS1 expression. The human breast cancer cell lines MCF7 and T47D were stably transfected with CBP, SRC1, or control (con) expression vectors. CBP, SRC1, NBS1, and β-actin expression was determined by western blot analysis.

of p21 which sustained Rb hyperphosphorylation allowing increased cell cycle progression in irradiated cells. These studies demonstrate important control of cell cycle and apoptosis by E2 and NBS1 in human breast cancer cells.

Our results demonstrated that induction of c-myc by E2 and p53 by IR was required for increased NBS1 expression in ER+ human breast cancer cell lines. The lack of NBS1 induction by E2 or IR alone may be due to the proximity of the myc and p53 response elements in the NBS1 intron 1 (20). p53 has been shown to bind to half sites in target gene promoters (21). Myc or p53 overexpression alone was not sufficient to induce NBS1 expression, but myc expression could substitute for E2 in irradiated cells. A previous report demonstrated that ER could bind directly to p53 and repress the function of the tumor suppressor (22). This interaction may provide an additional mechanism by which activated ER may inactivate p53 to facilitate cell cycle progression and inhibit apoptosis.

Our results demonstrated that CBP and SRC1 coactivators were recruited to the myc and p53 response elements in the NBS1 intron 1 and were sufficient to activate gene expression. Previous studies demonstrated that SRC1 physically interacts with p53 and potentiated p53 mediated transactivation (23). The coactivators CBP/p300 associate with and acetylate p53, and results in acetylation of histones in p53 target gene promoters (24-26). These studies demonstrate the importance of coactivator function in mediating the effects of DNA damage response in human breast cancer cells.

Mutations in the NBS1 gene have been associated with increased risk of breast cancer (9,10,27,28). Persistent radiation induced NBS1 foci has been associated with chromosomal instability and increased breast cancer risk (13). In mice, NBS1 null mutation is embryonic lethal but heterozygosity renders mice susceptible to tumor formation (5). However, mammary tumors are uncommon in mouse strains with reduced NBS1 function (6). Defects in cellular proliferation were noted in the cells of NBS1 deficient mice in previous studies (7). Loss of p53 has been shown to greatly increase tumorigenesis in NBS1 mutant mice, suggesting that p53 mediated DNA damage response may be responsible for apoptosis and increased tumor latency (29). A previous study demonstrated nuclear export of NBS1 following ionizing radiation as a mechanism of downregulating the DNA damage response (30). Loss of NBS1 has been shown to induce supernumerary centrosomes similar to those observed in BRCA1 deficient cells, leading to increased chromosomal instability (31). These studies demonstrate that impaired NBS1 function can result in cellular proliferation defects leading to increased tumor latency. It is interesting to speculate that tumorigenic clones that escape defective proliferation may be more aggressive and metastatic due to chromosomal aberrations induced by diminished NBS1 function.

Acknowledgements

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SUPPORTING DATA

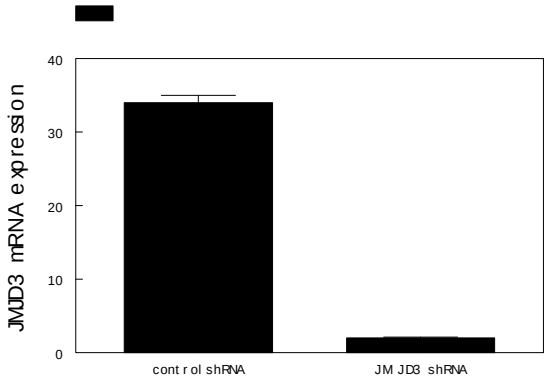


Fig. 1. JMJD3 mRNA expression in control and JMJD3 shRNA transduced ER+ cells from MMTV-Wnt1 tumors. Error bars indicate SEM from three independent tumor sets.

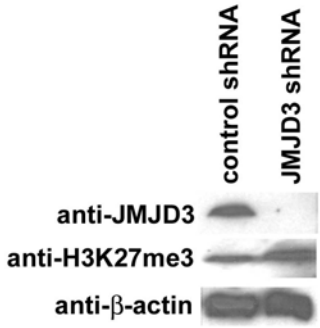
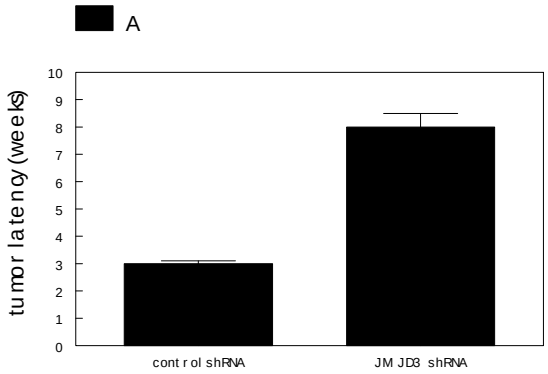


Fig. 2. Increased H3K27me3 levels in JMJD3 vs. control shRNA transduced ER+ cells from MMTV-Wnt1 tumors. β-actin expression is shown as loading controls for western blot. Representative blots are shown.



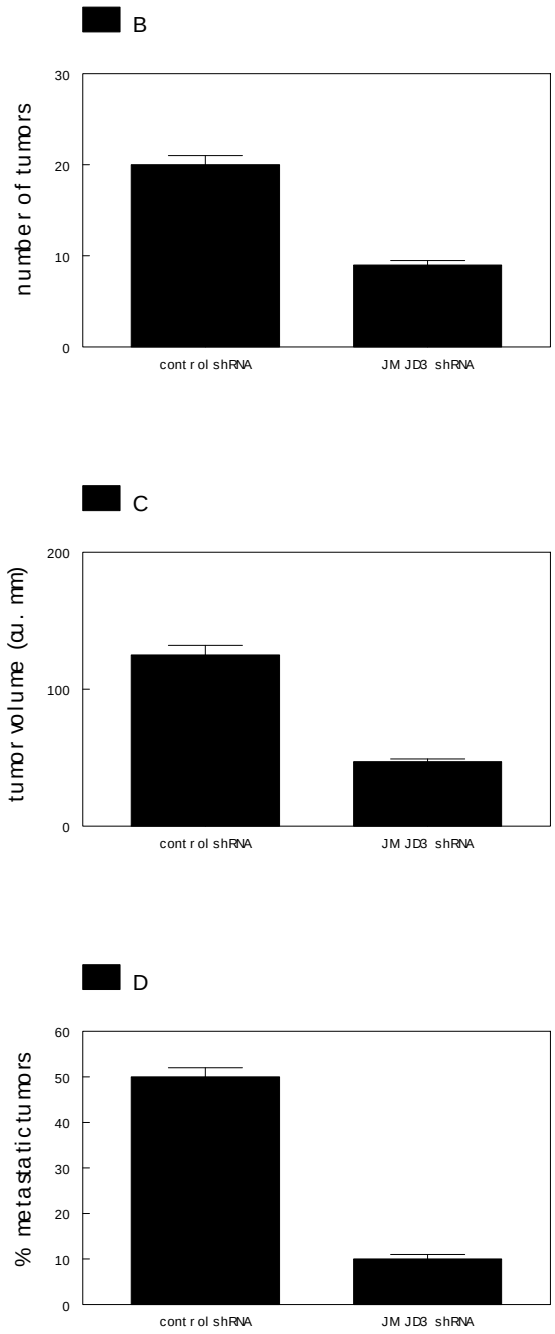


Fig. 3. JMJD3 inhibition in ER+ tumor cells co-transplanted with MMTV-Wnt1 MSC increases tumor latency, decreases tumor number, volume, and metastasis. Error bars indicate SEM of 20 independent tumor cell transplants.

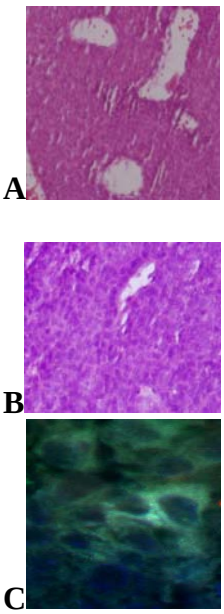


Fig. 4. Histopathologic analysis revealed poorly differentiated adenocarcinoma in both JMJD3 (A) and control (B) shRNA transduced ER+ cells co-transplanted with MMTV-Wnt1 MSC. (C) MSC demonstrated by double CD24/CD49f immunofluorescence. Representative sections are shown.

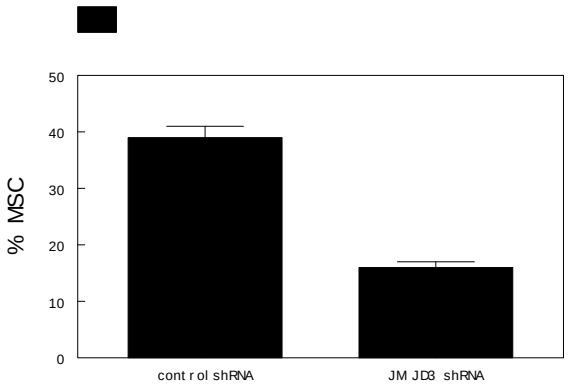


Fig. 5. Reduced MSC fraction in JMJD3 shRNA transduced ER+ tumor cells co-transplanted with MMTV-Wnt1 MSC. Error bars indicate SEM from 20 independent transplants.